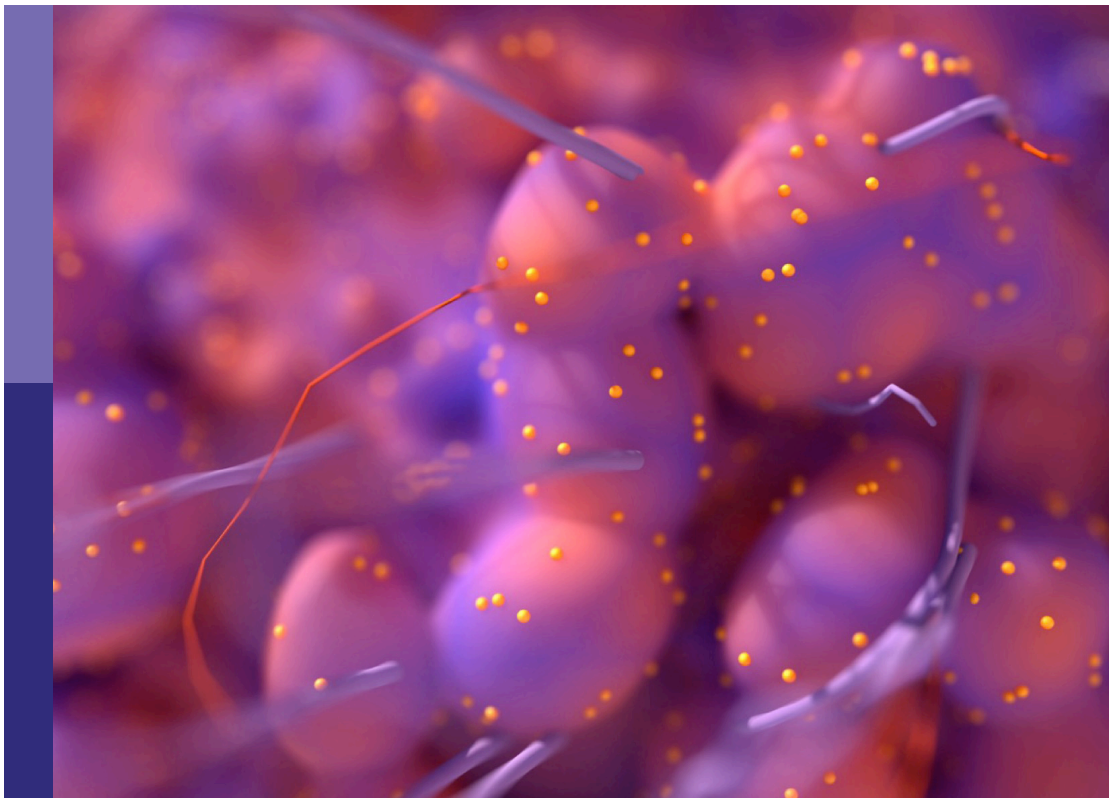
frontiers | Research Topics

The characteristics of pediatric soft tissue sarcomas: Recent advances in management and treatment

Edited by
Jilong Yang and Shunbin Xiong

Published in
Frontiers in Oncology



ISSN 1664-8714
ISBN 978-2-8325-3282-9
DOI 10.3389/978-2-8325-3282-9

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

The characteristics of pediatric soft tissue sarcomas: Recent advances in management and treatment

Topic editors

Jilong Yang — Tianjin Medical University Cancer Institute and Hospital, China

Shunbin Xiong — University of Texas MD Anderson Cancer Center, United States

Citation

Yang, J., Xiong, S., eds. (2023). *The characteristics of pediatric soft tissue sarcomas: Recent advances in management and treatment*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3282-9

Table of contents

04 Risk Factors for Metastasis at Initial Diagnosis With Ewing Sarcoma
Conglin Ye, Min Dai and Bin Zhang

11 Dysregulated m6A-Related Regulators Are Associated With Tumor Metastasis and Poor Prognosis in Osteosarcoma
Jianhao Li, Benchen Rao, Jie Yang, Liwen Liu, Maoxin Huang, Xin Liu, Guangying Cui, Chao Li, Qicai Han, Hao Yang, Xichun Cui and Ranran Sun

24 Targeting Tumor-Associated Macrophages in the Pediatric Sarcoma Tumor Microenvironment
Jane Koo, Masanori Hayashi, Michael R. Verneris and Alisa B. Lee-Sherick

43 Osteosarcoma and Metastasis
Gaohong Sheng, Yuan Gao, Yong Yang and Hua Wu

70 Application of Multi-Omics Approach in Sarcomas: A Tool for Studying Mechanism, Biomarkers, and Therapeutic Targets
Zijian Zou, Wei Sun, Yu Xu, Wanlin Liu, Jingqin Zhong, Xinyi Lin and Yong Chen

81 Osteosarcoma immune prognostic index can indicate the nature of indeterminate pulmonary nodules and predict the metachronous metastasis in osteosarcoma patients
Xuanhong He, Minxun Lu, Xin Hu, Longqing Li, Chang Zou, Yi Luo, Yong Zhou, Li Min and Chongqi Tu

95 Prognostic factors of pediatric pelvic and genitourinary rhabdomyosarcoma: An analysis based on SEER database
Jiheng Wu, Xinyi Shou, Jiabin Cai, Junqing Mao, Jianqin Qian, Jinhu Wang and Shaoqing Ni

107 Clinical features and therapeutic outcomes of alveolar soft part sarcoma in children: A single-center, retrospective study
Zhichao Tan, Jiayong Liu, Ruifeng Xue, Zhengfu Fan, Chujie Bai, Shu Li, Tian Gao, Lu Zhang and Xinyu Wang

114 Identification and validation of a novel ubiquitination-related gene *UBE2T* in Ewing's sarcoma
Guoxin Qu, Yuanchun Xu, Ye Qu, Jinchao Qiu, Guosheng Chen, Nannan Zhao and Jin Deng

127 Haploidentical hematopoietic stem cell transplantation as individual treatment option in pediatric patients with very high-risk sarcomas
Thomas Eichholz, Michaela Döring, Stefano Giardino, Bernd Gruhn, Christian Seitz, Tim Flaadt, Wolfgang Schwinger, Martin Ebinger, Ursula Holzer, Markus Mezger, Heiko-Manuel Teltschik, Monika Sparber-Sauer, Ewa Koscielniak, Michael Abele, Rupert Handgretinger and Peter Lang

frontiers in Oncology

ORIGINAL RESEARCH
published: 16 October 2019
doi: 10.3389/fonc.2019.01043

Risk Factors for Metastasis at Initial Diagnosis With Ewing Sarcoma

Conglin Ye, Min Dai†* and *Bin Zhang*†*

Department of Orthopedics, Artificial Joints Engineering and Technology Research Center of Jiangxi Province, The First Affiliated Hospital of Nanchang University, Nanchang, China

Purpose: We aimed to identify potential risk factors predictive of metastasis at initial diagnosis in Ewing sarcoma patients.

Patients and methods: We enrolled selected patients diagnosed with Ewing sarcoma between 2004 and 2015 in the Surveillance, Epidemiology, and End Results (SEER) Program database. Demographic and clinical features of patients were analyzed to demonstrate the potential risk factors of distant metastasis at presentation. We utilized descriptive statistics, univariate methods, and a series of regression models to analyze the significance of risk factors. Moreover, we conducted survival analysis in patients with different metastatic sites through Kaplan–Meier analysis.

Results: We identified 1,066 cases of Ewing sarcoma and 332 (31.1%) of the patients had metastasis at initial diagnosis. In the univariate logistic regression analysis, patients had higher probability of metastasis at initial diagnosis if they aged between 18 and 59 years old (OR = 1.43; 95% CI, 1.09 to 1.86), had a tumor located in the axial or cranial bones (OR = 1.38; 95% CI, 1.05 to 1.81), or had a tumor over 8 cm (OR = 2.55; 95% CI, 1.66 to 3.89). These three factors were still significant when analyzed in a multivariate logistic regression model or another multivariate logistic regression model controlling for age, location, and tumor size, which had univariate $p < 0.1$. Besides, we found that patients with lung metastasis alone had a better prognosis than patients with bone metastasis alone or with two or more metastatic sites ($p < 0.01$).

Conclusion: Ewing sarcoma patients with an age between 18 and 59 years old, a tumor in the axial or cranial bones, and a tumor size over 8 cm had an increased likelihood to have metastatic diseases at initial diagnosis.

Keywords: Ewing sarcoma, metastatic disease, SEER, tumor size, survival

OPEN ACCESS

Edited by:
Elizabeth R. Lawlor, University of Michigan Health System, United States

Reviewed by:
Mark Krailo, University of Southern California, United States
Joanna Kitlinska, Georgetown University, United States

***Correspondence:**
Min Dai
daimin@medmail.com.cn
Bin Zhang
13970823907@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *14 June 2019*
Accepted: *25 September 2019*
Published: *16 October 2019*

Citation:
Ye C, Dai M and Zhang B (2019) Risk Factors for Metastasis at Initial Diagnosis With Ewing Sarcoma. Front. Oncol. 9:1043. doi: 10.3389/fonc.2019.01043

INTRODUCTION

Ewing sarcoma (ES) is the second most common primary malignant bone tumor in children and young people, following osteosarcoma (1). Owing to the advance in surgery, radiation, and multidrug chemotherapy in the last few decades, the 5 year overall survival rate of the patients with localized ES has been improved to nearly 75% (2). However, the 5 year survival rate of patients with metastasis is only 20–45%, depending on the metastatic sites (3). It is reported that approximately 25% of ES patients have metastatic diseases at initial diagnosis (4). So far, little is known about risk factors related to higher odds of metastasis at initial diagnosis in ES patients.

Due to the rarity of ES, obtaining adequate cases from our clinical practice to conduct the current research is extremely difficult. Thus, we used the SEER Program database, a commonly used tool to study rare tumors, which provides data from 17 geographically variable cancer registries and involves about 26% of the United States population.

We carried out the current study to identify risk factors of distant metastasis at initial diagnosis in ES patients in both demographic data (age, sex, and race) and tumor characteristics (location and size).

MATERIALS AND METHODS

Ethics Statement

This research was approved by the Ethics Committee of our institution. Since neither human subjects nor personal private information was involved in the data, informed consent from the patients was not required for this study.

Patient Population

We identified all the ES cases recorded in the SEER database from 2004 to 2015, utilizing the SEER*Stat software (version 8.3.5). We included a total of 1,066 selected cases in this study, as shown in **Figure 1**.

Among these patients with ES, those with one primary tumor and clear metastatic status at presentation were enrolled in our research. This study focused solely to the bone tumors and extra-osseous ES are not included. Moreover, we excluded patients with multiple primary tumors and those with unknown metastatic status.

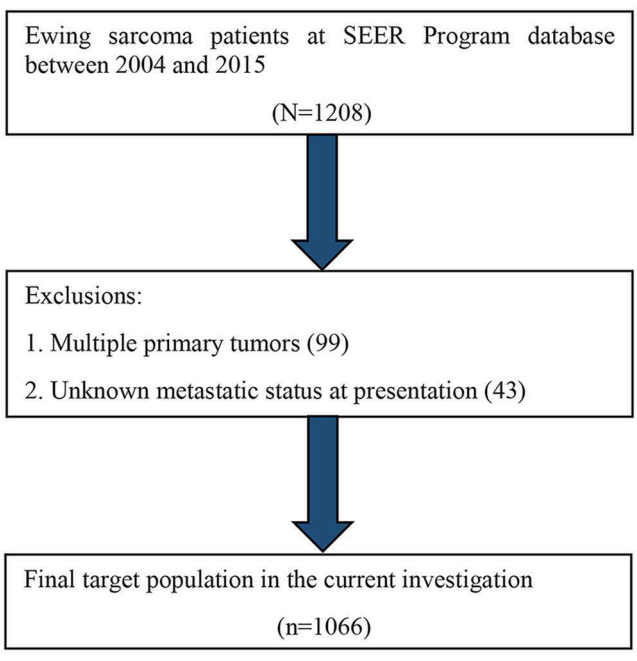


FIGURE 1 | Flow diagram of the target patient population selected from the SEER database.

We studied demographic features including age, sex, and race. Patient age in the SEER database begins at 0 years and ends at 85 years or more in 5 year intervals. A previous study found that ES patients ≥40 years at diagnosis have a higher possibility to have extraosseous tumors, metastasis, and a lower survival rate (5). Most ES patients are juveniles and there is strong evidence that patients aged 17 years old or less at diagnosis are at reduced risk for death. Thus, in this study, we divided the patients into three age groups of zero to seventeen years old (0–17 years), eighteen to fifty-nine years old (18–59 years), and sixty to eighty-five years old or older (60–85+ years) based on their age at diagnosis. We categorized sex as male or female. The race was characterized as white, black, other (American Indian/AK Native, Asian/Pacific Islander), or unknown.

We also had great interest in tumor-related factors including primary site and tumor size. The primary site in the SEER database is considerably vague, and we could not confirm the explicit bone or the precise site in the bone. Thus, we classified the primary site as the extremity bones (long and short bones of the extremities), axial or cranial bones (pelvis, spine, ribs, mandible, and skull), or unknown sites, similar to what has been done previously (6–8).

We recorded tumor size as a continuous variable. The patients were divided into four size groups of less than 5 centimeters (≤5 cm), between 5 and 8 centimeters (>5 to 8 cm), over 8 centimeters (>8 cm), or unknown size, according to previous investigations (5, 7, 9).

Distant metastatic sites, including lung, bone, liver, and brain, have been recorded in the SEER database since 2010. Therefore, we utilized the data from 2010 to 2015 to carry out a survival analysis based on different metastatic sites. A total of 152 selected cases were included in the survival analysis, as shown in **Figure 4**.

Statistical Methods

We first investigated the total rate of distant metastasis at initial diagnosis among the 1066 patients with ES. Then, we utilized descriptive statistics and univariate methods to determine the percentage of patients with localized disease or metastasis based on the potential risk factors we proposed (age, race, sex, primary site, and tumor size). Lastly, we used several regression models to study the correlation among metastasis at initial diagnosis and a series of demographic and clinical features, including sex, age, race, primary site, and tumor size. Model 1 conducted univariate logistic regression analysis of all the possible risk factors in the 1,066 patients. Model 2 carried out a multivariate logistic regression analysis of all the potential risk factors. Model 3 conducted multivariate logistic regression analysis in variables

TABLE 1 | Ewing sarcoma with metastasis at diagnosis, 2004 to 2015.

	No.	Metastasis at diagnosis no. (%)
Total	1,066	332 (31.1)

No., number; no., number.

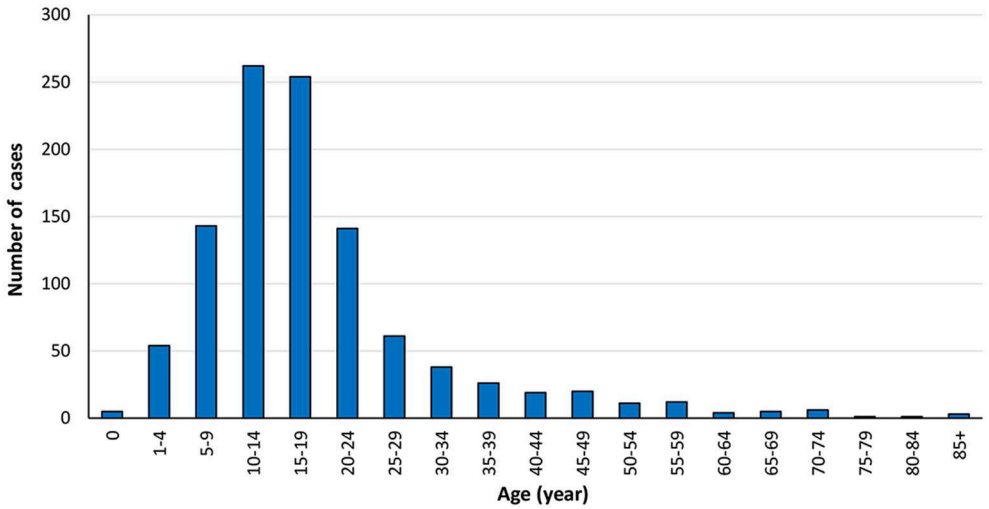


FIGURE 2 | The number of Ewing sarcoma cases from 2004 to 2015 according to age at diagnosis.

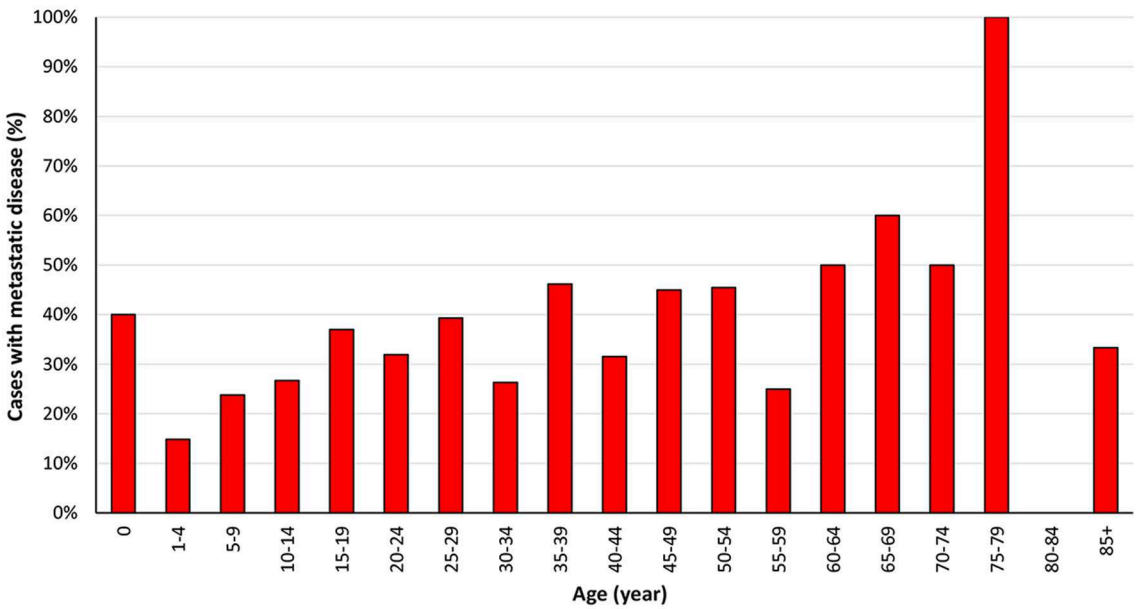


FIGURE 3 | Percentage of Ewing sarcoma cases with metastasis at initial diagnosis from 2004 to 2015 according to age at diagnosis.

with univariate $p < 0.1$. We used the log-rank test to evaluate the association between metastatic sites and ES-related survival. $p < 0.05$ was considered statistically significant. We executed all the statistical analysis via SPSS 17.0 software.

Missing Data

We found missing data in race, primary site, and tumor size. 4/1,066 (0.38%) patients had a missing race variable. 33/1,066 (3.1%) patients had a missing tumor site variable. 263/1,066 (24.7%) patients had a missing tumor size variable. When these predictor variables with missing data were applied in univariate analysis or regression models, we categorized patients with missing data as unknown for statistical analysis.

RESULTS

We included 1,066 ES cases diagnosed from 2004 to 2015 in the present research. The total proportion of distant metastasis at initial diagnosis was 31.1%, as shown in **Table 1**. Most of the 1,066 cases occurred in children, adolescents, and young people, which consists with previous research (**Figure 2**) (7). The ratio of ES patients with metastasis at presentation varied according to the age (**Figure 3**). Distant metastasis at initial diagnosis was

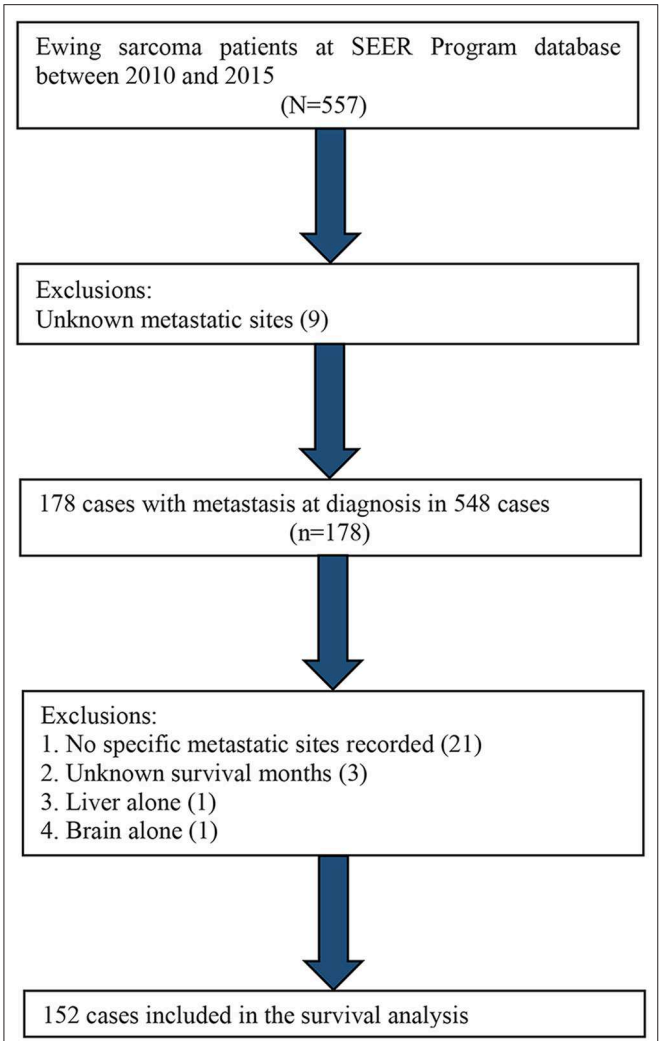


FIGURE 4 | Flow diagram of the patient population selected from the SEER database for the survival analysis.

TABLE 2 | Univariate analysis of patient characteristics and metastasis at diagnosis with Ewing sarcoma, 2004 to 2015.

Category	No.	Metastasis at diagnosis no. (%)	*p*-value
Age in years			0.006
0–17	634	176 (27.8)	
18–59	412	146 (35.4)	
60–85+	20	10 (50.0)	
Sex			0.459
Male	673	215 (31.9)	
Female	393	117 (29.8)	
Race			0.301
White	941	294 (31.2)	
Black	40	16 (40.0)	
Other	81	20 (24.7)	
Unknown	4	2 (50.0)	
Location			<0.001
Extremity	472	124 (26.3)	
Axial	561	185 (33.0)	
Unknown	33	23 (69.7)	
Size			<0.001
≤5 cm	192	34 (17.7)	
>5 to 8 cm	221	56 (25.3)	
>8 cm	390	138 (35.4)	
Unknown	263	104 (39.5)	

No., number; no., number.

more frequent among patients aged 18–59 years old (35.4%) than patients younger than 18 years old (27.8%) ($p = 0.006$) (**Table 2**). We also found that axial or cranial primary tumor site and a tumor size larger than 5 cm was related to an elevated rate of metastasis at diagnosis ($p < 0.001$). We found no significant difference in the rate of metastatic disease at diagnosis among patients with different sex ($p = 0.459$) or race ($p = 0.301$).

The Model 1 univariate logistic regression analysis of all the variables indicated raised likelihood of metastasis at diagnosis among patients aged between 18 and 59 years old (OR = 1.43; 95% confidence interval [CI], 1.09 to 1.86), patients had a tumor located in the axial or cranial bones (OR = 1.38; 95% CI, 1.05 to 1.81), and patients with a tumor size over 8 cm (OR = 2.55; 95% CI, 1.66 to 3.89) (**Table 3**). The Model 3 multivariate logistic regression analysis, which contained all the variables with univariate $p < 0.1$, also showed increased incidence of metastasis at initial diagnosis among patients aged between 18 and 59 years old (OR = 1.38; 95% CI, 1.05 to 1.82), patients had a tumor located in the axial or cranial bones (OR = 1.42; 95% CI, 1.07 to 1.87), and patients with a tumor size over 8 cm (OR = 2.86; 95% CI, 1.85 to 4.44). The Model 2 multivariate logistic regression analysis of all the variables was carried out to verify the stability of our findings. Model 2 indicated a consistent result with the other two models.

Table 4 shows the distributions of distant metastatic sites. The most common ES metastatic sites were lung, followed by bone, liver, and brain. We excluded patients with no specific metastatic sites ($n = 21$), unknown survival months ($n = 3$), metastasis in liver alone ($n = 1$), and metastasis in brain alone ($n = 1$). The remaining cases were used in the Kaplan–Meier analysis. The Kaplan–Meier curve revealed that patients with lung metastasis alone had a better outcome than patients with bone metastasis alone or patients with two or more metastatic sites ($p < 0.01$) (**Figure 5**).

DISCUSSION

In this study, we found that 31.1% of ES patients had distant metastasis at initial diagnosis. Age between 18 and 59 years old, axial or cranial tumor sites, and tumor size larger than 8 cm were related to increased odds of distant metastasis at initial diagnosis. Besides, we discovered that patients with lung metastasis alone had better tumor-specific survival rate than patients with bone metastasis alone or patients with two or more metastatic sites.

TABLE 3 | Odds ratios for risk of presentation with metastatic disease*.

Variable	Model 1[a]	Model 2[b]	Model 3[c]
Cases included	1,066	1,066	1,066
Age in years			
0–17	Ref	Ref	Ref
18–59	1.43 (1.09–1.86)	1.37 (1.04–1.81)	1.38 (1.05–1.82)
60–85+	2.60 (1.07–6.36)	2.03 (0.76–5.43)	2.04 (0.76–5.45)
Sex			
Male	Ref	Ref	–
Female	0.90 (0.69–1.18)	0.97 (0.74–1.29)	–
Race			
White	Ref	Ref	–
Black	1.47 (0.77–2.80)	1.36 (0.70–2.66)	–
Other	0.72 (0.43–1.22)	0.76 (0.45–1.31)	–
Unknown	2.20 (0.31–15.70)	2.88 (0.39–21.39)	–
Location			
Extremity	Ref	Ref	Ref
Axial	1.38 (1.05–1.81)	1.43 (1.08–1.88)	1.42 (1.07–1.87)
Unknown	6.46 (2.99–13.94)	6.04 (2.67–13.68)	6.01 (2.66–13.59)
Size			
≤5 cm	Ref	Ref	Ref
>5 to 8 cm	1.58 (0.98–2.55)	1.74 (1.07–2.85)	1.76 (1.08–2.87)
>8 cm	2.55 (1.66–3.89)	2.86 (1.84–4.43)	2.86 (1.85–4.44)
Unknown	3.04 (1.95–4.75)	3.14 (1.98–4.97)	3.17 (2.01–5.02)

The values are given as the odds ratio, with the 95% confidence interval in parentheses.
[a] *Univariate logistic regression analysis of all categorical variables.*
[b] *Multivariate logistic regression analysis includes all categorical variables.*
[c] *Multivariate logistic regression analysis includes categorical variables with univariate $p < 0.1$.*
Ref, reference.

TABLE 4 | The distribution of distant metastatic sites.

Specific site of distant metastasis	*n*	Percentage
Lung alone	72	45.9%
Bone alone	49	31.2%
Liver alone	1	0.6%
Brain alone	1	0.6%
≥2 sites	34	21.7%

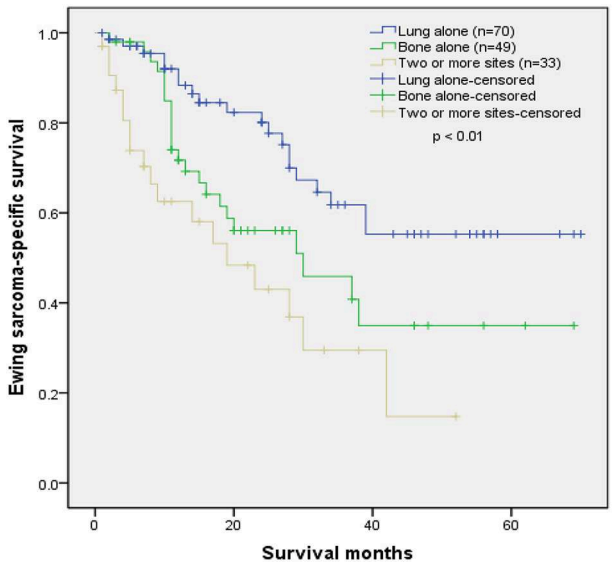


FIGURE 5 | Kaplan–Meier curve of Ewing sarcoma-specific survival according to the metastatic sites.

Previous researches have demonstrated that metastasis at initial diagnosis was an independent predictive factor of poorer overall survival (7, 10–13). Ramkumar et al. found that advanced age, axial tumor location, and larger tumor size were associated with increased odds of detectable metastatic disease at initial diagnosis in patients with Ewing family of tumors (EFT) (14). The current study investigated specifically bone Ewing sarcoma rather than the EFT. To our knowledge, there are few previous researches regarding risk factors for metastasis at initial diagnosis in ES patients. We tried to provide new insights into the predictive factors of distant metastasis at initial diagnosis. Firstly, it included a large sample that was a representative population of the United States. Secondly, we analyzed not only demographic features but also clinical characteristics. Finally, we utilized several multivariate regression models to verify our findings repeatedly. Taken together, we determined several risk factors and therefore helped identify susceptible ES patient groups for metastasis at initial diagnosis.

A few previous researches have identified relevance between older age and a poorer prognosis in ES patients. Karski et al. reported that patients over 40 years old diagnosed with ES were more probable to have metastasis. Moreover, they found that older patients had a lower survival rate (5). Huh et al. also determined patients younger than 10 years old with ES family of tumors had better overall survival rate than older patients (11). In this study, age between 18 and 59 years old was an independent risk factor for metastasis at presentation. Patients younger than 18 years old had lower odds of metastasis at initial diagnosis ($p < 0.01$).

We also determined that an axial or cranial tumor site and tumor size larger than 8 cm contributed to metastasis at initial diagnosis in ES. Some prior researches on ES also showed that tumors in the axial bones and larger tumor size were closely related to a poorer prognosis. For instance, Duchman et al. found that ES patients with metastasis at initial diagnosis, axial tumor site, and tumor larger than 10 cm had lower cause-specific survival rate at 10 years (7). Lee et al. confirmed that older age, metastasis, and larger tumor size were predictive for poor overall survival rate in ES patients (15). The dismal outcomes in these patients could be partly explained by the difficulty in conducting sufficient surgical resection and acquiring proper margins (7, 8, 16). Argon et al. reported that ES originating from the axial bones had a worse outcome than those at the extremities owing to frequent recurrence, fast distant metastasis,

larger tumor volume, and difficulties in the surgical intervention (17). Moreover, tumors in the axial bones were usually closer to large vessels, which may elevate the possibility of distal metastatic diseases (18–20). Besides, patients with tumors in the axial bones usually lacked palpable masses or dramatic symptoms. Thus, tumors in the axial bones may also be observed and detected later, which may possibly lead to delayed diagnosis and elevated odds of distant metastasis (7, 8). A larger tumor size also implied increased time before diagnosis and more blood vessels involved. Meanwhile, tumor cells continued to divide uncontrollably over time. These might facilitate metastatic diseases at initial diagnosis due to larger tumor size. In the present study, we merged patients with cranial ES and axial ES into the axial or cranial location category for statistical analysis. The result was similar to previous studies. Cotterill et al. demonstrated that there was a trend for better survival for patients with lung involvement compared with patients with bone metastases or a combination of lung and bone for the ES patients with metastases (13). In this research, we came to a consistent conclusion that patients with lung metastasis alone had a better prognosis than patients with bone metastasis alone or patients with two or more metastatic sites.

Although the present research did not probe into treatment guidance or prognostic factors, our findings did have some important clinical significance. With the awareness of these high-risk factors, doctors can inform certain patient groups about the high possibility of metastasis at initial diagnosis. Patients with high-risk factors might benefit from more frequent and cautious pulmonary surveillance or screening examinations at early stage. Early diagnosis and early treatment could obtain better outcomes. Besides, according to the different metastatic sites, the doctor could partly predict the prognosis of ES patients.

However, the present research had several limitations. Firstly, though the SEER database provided numerous cases to analyze, it did have some inevitable restrictions. We could not verify the diagnostic accuracy of metastasis. Besides, we could not acquire exact information about tumor size or precise location of the tumors. Secondly, we did not investigate socioeconomic factors such as income, poverty, or education status of the patients. Thirdly, we did not examine the survival status in patients with liver or brain metastasis alone. Finally, we did not study the treatment methods or prognostic factors. These were not the goal of the current research, but they represented a crucial part for further exploration.

CONCLUSIONS

In short, the present study demonstrated that age between 18 and 59 years old, tumor located in the axial or cranial skeleton, and tumor size $>$ 8 cm were closely related to a greater likelihood of distant metastasis at initial diagnosis in patients with ES. Additionally, patients with lung metastasis alone had a better prognosis than patients with bone metastasis alone or patients with two or more metastatic sites.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. These data can be found here: https://seer.cancer.gov/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committee of The First Affiliated Hospital of Nanchang University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CY, MD, and BZ designed this study. CY performed the search and collected data. MD and BZ rechecked data. CY performed analysis and wrote the manuscript. All authors reviewed the manuscript.

FUNDING

This study was supported by the Foundation of Health Department of Jiangxi Province (20175108&20175113&2016A073) and the Foundation of Education Department of Jiangxi Province (GJJ160127).

ACKNOWLEDGMENTS

We would like to thank the SEER database for the collection of clinical data.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* (2011) 61:69–90. doi: 10.3322/caac.20107
2. Balamuth NJ, Womer RB. Ewing's sarcoma. *Lancet Oncol.* (2010) 11:184–92. doi: 10.1016/S1470-2045(09)70286-4
3. Gaspar N, Hawkins DS, Dirksen U, Lewis IJ, Ferrari S, Le Deley MC, et al. Ewing sarcoma: current management and future approaches through collaboration. *J Clin Oncol.* (2015) 33:3036–46. doi: 10.1200/JCO.2014.59.5256
4. Esiashvili N, Goodman M, Marcus RB Jr. Changes in incidence and survival of Ewing sarcoma patients over the past 3 decades: surveillance epidemiology and end results data. *J Pediatr Hematol Oncol.* (2008) 30:425–30. doi: 10.1097/MPH.0b013e31816e22f3
5. Karski EE, Matthay KK, Neuhaus JM, Goldsby RE, Dubois SG. Characteristics and outcomes of patients with Ewing sarcoma over 40 years of age at diagnosis. *Cancer Epidemiol.* (2013) 37:29–33. doi: 10.1016/j.canep.2012.08.006
6. Applebaum MA, Goldsby R, Neuhaus J, DuBois SG. Clinical features and outcomes in patients with Ewing sarcoma and regional lymph node involvement. *Pediatr Blood Cancer.* (2012) 59:617–20. doi: 10.1002/pbc.24053
7. Duchman KR, Gao Y, Miller BJ. Prognostic factors for survival in patients with Ewing's sarcoma using the surveillance, epidemiology, and

end results (SEER) program database. *Cancer Epidemiol.* (2015) 39:189–95. doi: 10.1016/j.canep.2014.12.012
8. Miller BJ, Cram P, Lynch CF, Buckwalter JA. Risk factors for metastatic disease at presentation with osteosarcoma: an analysis of the SEER database. *J Bone Joint Surg Am.* (2013) 95:e89. doi: 10.2106/JBJS.L.01189
9. Liu Q, Luo D, Cai S, Li Q, Li X. Real-world implications of nonbiological factors with staging, prognosis and clinical management in colon cancer. *Cancers.* (2018) 10:E263. doi: 10.3390/cancers10080263
10. Hamilton SN, Carlson R, Hasan H, Rassekh SR, Goddard K. Long-term outcomes and complications in pediatric Ewing sarcoma. *Am J Clin Oncol.* (2017) 40:423–8. doi: 10.1097/COC.0000000000000176
11. Huh WW, Daw NC, Herzog CE, Munsell MF, McAleer MF, Lewis VO. Ewing sarcoma family of tumors in children younger than 10 years of age. *Pediatr Blood Cancer.* (2017) 64:e26275. doi: 10.1002/pbc.26275
12. Lee CY, Yen CC, Yen HJ, Shiau CY, Chao TC, Wu PK, et al. Outcomes of 50 patients with Ewing sarcoma family of tumors treated at a single institution in Taiwan. *Medicine.* (2016) 95:e3830. doi: 10.1097/MD.0000000000003830
13. Cotterill SJ, Ahrens S, Paulussen M, Jurgens HF, Voute PA, Gadner H, et al. Prognostic factors in Ewing's tumor of bone: analysis of 975 patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group. *J Clin Oncol.* (2000) 18:3108–14. doi: 10.1200/JCO.2000.18.17.3108
14. Ramkumar DB, Ramkumar N, Miller BJ, Henderson ER. Risk factors for detectable metastatic disease at presentation in Ewing sarcoma - an analysis of the SEER registry. *Cancer Epidemiol.* (2018) 57:134–9. doi: 10.1016/j.canep.2018.10.013
15. Lee J, Hoang BH, Ziogas A, Zell JA. Analysis of prognostic factors in Ewing sarcoma using a population-based cancer registry. *Cancer.* (2010) 116:1964–73. doi: 10.1002/cncr.24937
16. Bacci G, Longhi A, Briccoli A, Bertoni F, Versari M, Picci P. The role of surgical margins in treatment of Ewing's sarcoma family tumors: experience of a single institution with 512 patients treated with adjuvant and neoadjuvant chemotherapy. *Int J Radiat Oncol Biol Phys.* (2006) 65:766–72. doi: 10.1016/j.ijrobp.2006.01.019
17. Argon A, Basaran M, Yaman F, Dizdar Y, Sakar B, Camlica H, et al. Ewing's sarcoma of the axial system in patients older than 15 years: dismal prognosis despite intensive multiagent chemotherapy and aggressive local treatment. *Jpn J Clin Oncol.* (2004) 34:667–72. doi: 10.1093/jjco/hyh122
18. Fahey M, Spanier SS, Vander Griend RA. Osteosarcoma of the pelvis. A clinical and histopathological study of twenty-five patients. *J Bone Joint Surg Am.* (1992) 74:321–30. doi: 10.2106/00004623-199274030-00002
19. Kawai A, Huvos AG, Meyers PA, Healey JH. Osteosarcoma of the pelvis. Oncologic results of 40 patients. *Clin Orthop Relat Res.* (1998) 348:196–207. doi: 10.1097/00003086-199803000-00030
20. Song K, Shi X, Liang X, Wang H, Zou F, Lu F, et al. Risk factors for metastasis at presentation with conventional chondrosarcoma: a population-based study. *Int Orthop.* (2018) 42:2941–8. doi: 10.1007/s00264-018-3942-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 02 June 2020
doi: 10.3389/fonc.2020.00769

Dysregulated m6A-Related Regulators Are Associated With Tumor Metastasis and Poor Prognosis in Osteosarcoma

Jianhao Li[1,2†], Benchen Rao[1,2†], Jie Yang[3†], Liwen Liu[1,2], Maoxin Huang[4], Xin Liu[1,2], Guangying Cui[1,2], Chao Li[5], Qicai Han[6], Hao Yang[6], Xichun Cui[2] and Ranran Sun[1,2*]*

[1] Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Key Laboratory of Clinical Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Orthopedics, Zhengzhou Central Affiliated Hospital to Zhengzhou University, Zhengzhou, China, [4] Dermatology Department, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Orthopedics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [6] Department of Bone and Soft Tissue, The Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Rimas J. Orentas, Seattle Children's Research Institute, United States

Reviewed by:
Songmei Liu, Wuhan University, China
Karina Formoso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

***Correspondence:**
Xichun Cui
xichun639@126.com
Ranran Sun
fccsunrr@zzu.edu.cn

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *27 December 2019*
Accepted: *21 April 2020*
Published: *02 June 2020*

Citation:
Li J, Rao B, Yang J, Liu L, Huang M, Liu X, Cui G, Li C, Han Q, Yang H, Cui X and Sun R (2020) Dysregulated m6A-Related Regulators Are Associated With Tumor Metastasis and Poor Prognosis in Osteosarcoma. Front. Oncol. 10:769. doi: 10.3389/fonc.2020.00769

Background: Osteosarcoma (OS) is the most common primary bone tumor. The disease has a poor prognosis due to the delay in the diagnosis and the development of metastasis. N6-Methyladenosine (m6A)-related regulators play an essential role in various tumors. In this study, a comprehensive analysis was conducted to elucidate the relationship between the expression profiles of m6A-related molecules and the clinical outcome of OS patients.

Materials and Methods: Public genome datasets and a tissue microarray (TMA) cohort were used to analyze the mRNA and protein expression levels of m6A regulators. Next, immunofluorescence (IF) analysis was used to determine the subcellular localization of m6A-related molecules. Kaplan–Meier and Cox regression analyses were performed to confirm the prognostic value of m6A-related molecules in OS. A comprehensive bioinformatic analysis was conducted to identify the potential molecular mechanisms mediated by m6A modification in OS.

Results: We found that m6A-related regulator expression was dysregulated in OS tissues, especially in metastatic tumor tissues. Low expression of METTL3, METTL14, and YTHDF2 and high expression of KIAA1429 and HNRNPA2B1 were significantly associated with poor prognosis in the TMA cohort. Simultaneously, the genome meta-cohort analysis revealed that low expression of FTO and METTL14 and high expression of METTL3, HNRNPA2B1, and YTHDF3 were associated with poor prognosis in OS. Cox regression analysis showed that HNRNPA2B1 might be an independent risk factor for OS. Bioinformatic analysis indicated that m6A regulators might be involved in OS progression through humoral immune response and cell cycle pathways.

Conclusion: M6A-related regulators are frequently dysregulated and correlate with metastasis and prognosis in OS. M6A-related regulators may serve as novel therapeutic targets and prognostic biomarkers for OS.

Keywords: N6-methyladenosine, osteosarcoma, tumor metastasis, prognosis, biomarkers

INTRODUCTION

Human osteosarcoma (OS) is one of the most common aggressive bone cancers, and it has higher incidence and mortality rates in teenagers; it frequently affects distal femur, proximal tibia, and humerus (1, 2). Despite significant progress in therapeutic strategies against OS over the past few decades, its prognosis remains poor due to the delay in the diagnosis and the development of metastasis (3). Thus, it is critical to clarify novel biomarkers and to ensure the effective treatment of OS patients.

N6-Methyl adenosine (m6A) is a posttranscriptional modification of RNA and is the most prevalent internal chemical modification of mRNAs (4). M6A network components have been well characterized into three subtypes: "writers," "readers," and "erasers" (5). The m6A modification is facilitated by writers and removed by erasers; moreover, it can recruit specific reader proteins (6). M6A-related regulators are involved in various physiological and pathological processes through the regulation of RNA stability, mRNA splicing and translation, and microRNA processing (7–9). Additionally, m6A is involved in the initiation and progression of cancers, including liver cancer, breast cancer, glioma, cervical cancer, colorectal cancer, and hepatoblastoma (10–15). However, the clinical value and potential mechanism of m6A-related regulators in OS are still unclear.

In this study, we evaluated the expression status and prognostic value of m6A-related proteins in OS based on public genome database analysis and tissue microarray (TMA) analysis. We found that dysregulated expression of several m6A-related proteins was frequently and closely associated with tumor metastasis and clinical outcomes in OS. Moreover, consensus clustering for m6A-related regulators was performed to identify the clusters with a better prognosis. Mechanistically, Gene Set Enrichment Analysis (GSEA) and Gene Set Variation Analysis (GSVA) indicated that the dysregulated m6A-related regulators might be associated with cell cycle and humoral immune response pathways. In conclusion, we revealed that m6A-related regulators play a critical role in the development of OS and proposed that m6A-related regulators could be potential therapeutic targets.

MATERIALS AND METHODS

Datasets

The RNA-seq transcriptome data and clinical information from four OS datasets (GSE12865, GSE21257, GSE42352, GSE39055, TARGET-OS) were collected from the GEO (http://www.ncbi.nlm.nih.gov/geo/) and TARGET (https://ocg.cancer.gov/programs/target) databases, based on the inclusion and exclusion criteria of the GEO and TARGET databases. Inclusion criteria: datasets involving human osteosarcoma and expression profiling by array. Exclusion criteria: datasets with a sample size smaller than 10 (2, 11, 16, 17). The characteristics of the datasets are presented in **Table S1**. Subsequently, three gene expression matrix files (GSE21257, GSE39055, TARGET-OS) with survival follow-up data were merged into one meta-cohort. Next, the "sva" package of the R software was used to remove the batch effect (**Figure S1**). A total of 175 patients with survival follow-up information were divided into higher and lower expression groups using the best cutoff value of the log-rank test. Kaplan–Meier survival curves were used to analyze survival rates. Univariate and multivariate analyses were conducted based on the Cox model. The raw data were analyzed by BRB-array tools as previously described (18).

Tissue Samples

Studies involving human samples were approved (approval number: 2011-KY-047) by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (ZZU cohort), Zhengzhou Central Affiliated Hospital of Zhengzhou University, and the Affiliated Cancer Hospital of Zhengzhou University. All legal guardians of the children signed an informed consent. OS samples from the three hospitals were combined to create the TMA cohort containing 120 OS tissues and 65 surrounding non-tumorous tissues.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) of m6A-related genes was performed as described previously (19, 20). Briefly, 5-μm-thick TMA sections were deparaffinized and then treated with hydrogen peroxide to quench endogenous peroxidase activity. Subsequently, the sections were incubated overnight with anti-human m6A-related protein antibodies (1:200, Abcam, USA) at 4°C. Next, the immunoreactive cells were detected by SignalStain® DAB (CST, USA), then counterstained with Haematoxylin QS (Vector Laboratories). Two experienced pathologists, who were blinded to the clinicopathological data, evaluated the immunostaining samples separately. A semi-quantitative scoring system was established based on different staining intensities, and the proportion of positive cells was scored as follows: 0, none; 1+, <25%; 2+, 25–50%; 3+, 50–75%; and 4+, 75–100%. The staining intensity was scored as follows: 0, none; 1+, weak; 2+, medium; and 3+, strong. The total score was calculated by multiplying two subscores, and the samples with scores of 0–6 were regarded as low expression, whereas the other ratings were regarded as high expression during statistical analysis. Antibody information is listed in **Table S2**.

Cell Lines and Culture

The human OS cell lines U2-OS and KHOS-240S were purchased from ATCC (Manassas, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY, USA) and 100 U/mL penicillin/streptomycin (Corning, New York, NY, USA). The STR reports for the cell lines U2-OS and KHOS-240S are presented in Supplemental Materials 1–2.

Immunofluorescence Assay

OS cells cultured in 24-well plates were fixed in 4% paraformaldehyde and permeabilized in 1% Triton X-100 phosphate-buffered saline (PBS). After blocking with 1% bovine serum albumin (BSA), the cells were incubated with primary antibodies at 4°C overnight and then with appropriate

corresponding secondary antibodies (Jackson ImmunoResearch Inc., USA) at room temperature for 30 min. Finally, the nuclei were counterstained with DAPI (Beyotime, China), and images were obtained with a Zeiss Axio microscope (Zeiss, Oberkochen, Germany). Detailed information on the antibodies used in this study is listed in **Table S2**.

Statistical Analysis

All statistical analyses were performed using the R statistical package (R version 3.5.3) unless otherwise stated. The differences between the two independent groups were analyzed using Student's *t* test (unpaired, two-tailed) or Permutation test when there were fewer than three samples in either group (21). Kaplan–Meier overall survival analysis was performed with a log-rank test. Univariate Cox regression analysis was used to indicate the relationship between the different variables and survival. The correlation was evaluated using the two-tailed Pearson test. We clustered OS patients into different clusters with "ConsensusClusterPlus" (22). Additionally, 150 clinically actionable genes were obtained from a recent publication (23). Subsequently, the protein–protein interactions among m6A regulators and 150 clinically actionable genes were identified based on the STRING (https://string-db.org/) interaction database (24). Cytoscape software was used to

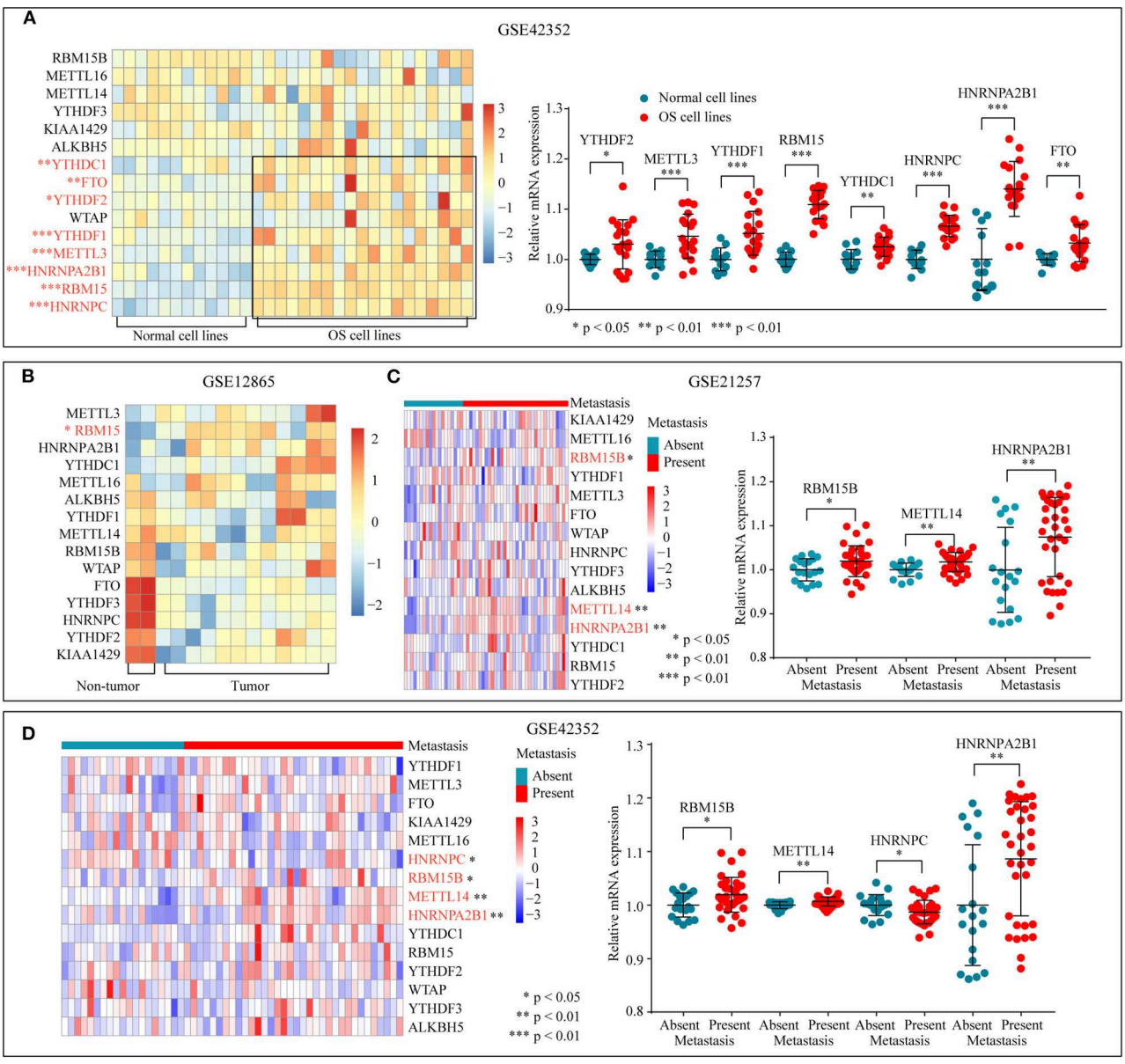


FIGURE 1 | m6A-related regulators expression status in osteosarcoma. **(A)** The mRNA expression level of m6A-related regulators in normal and OS cell lines of GSE42352. **(B)** The mRNA expression level of m6A-related molecules in tumor and non-tumor tissues of GSE12865. **(C,D)** The correlation between the mRNA expression of m6A-related regulators and tumor metastasis.

visualize the interactions. GSVA was performed using the Bioconductor R package "GSVA" (25). GSEA was conducted using clusterProfiler, an R/Bioconductor package (26). In all cases, $P < 0.05$ was considered statistically significant expression is frequently dysregulated.

RESULTS

M6A-Related Gene Expression Is Frequently Dysregulated in Osteosarcoma

To determine the significant biological function of m6A-related regulators in tumorigenesis and development, the expression pattern of m6A-related genes was analyzed through the GEO database (GSE42352) (**Figure 1A**). The mRNA expression levels of YTHDF2, YTHDF1, HNRNPC, FTO, METTL3, RBM15, HNRNPA2B1, and YTHDC1 were higher in OS cells than in normal cell lines. In addition, differential expression analysis of GSE12865 through a permutation test further confirmed that the expression of RBM15 was significantly upregulated in the tumor tissues (**Figure 1B**). Subsequently, the relationship between tumor metastasis and the expression level of m6A-related regulators was examined in GSE21257 and GSE42352 (**Figures 1C,D**). The overexpression of RBM15B, METTL14, and HNRNPA2B1 is significantly related to tumor metastasis. In conclusion, these results suggested

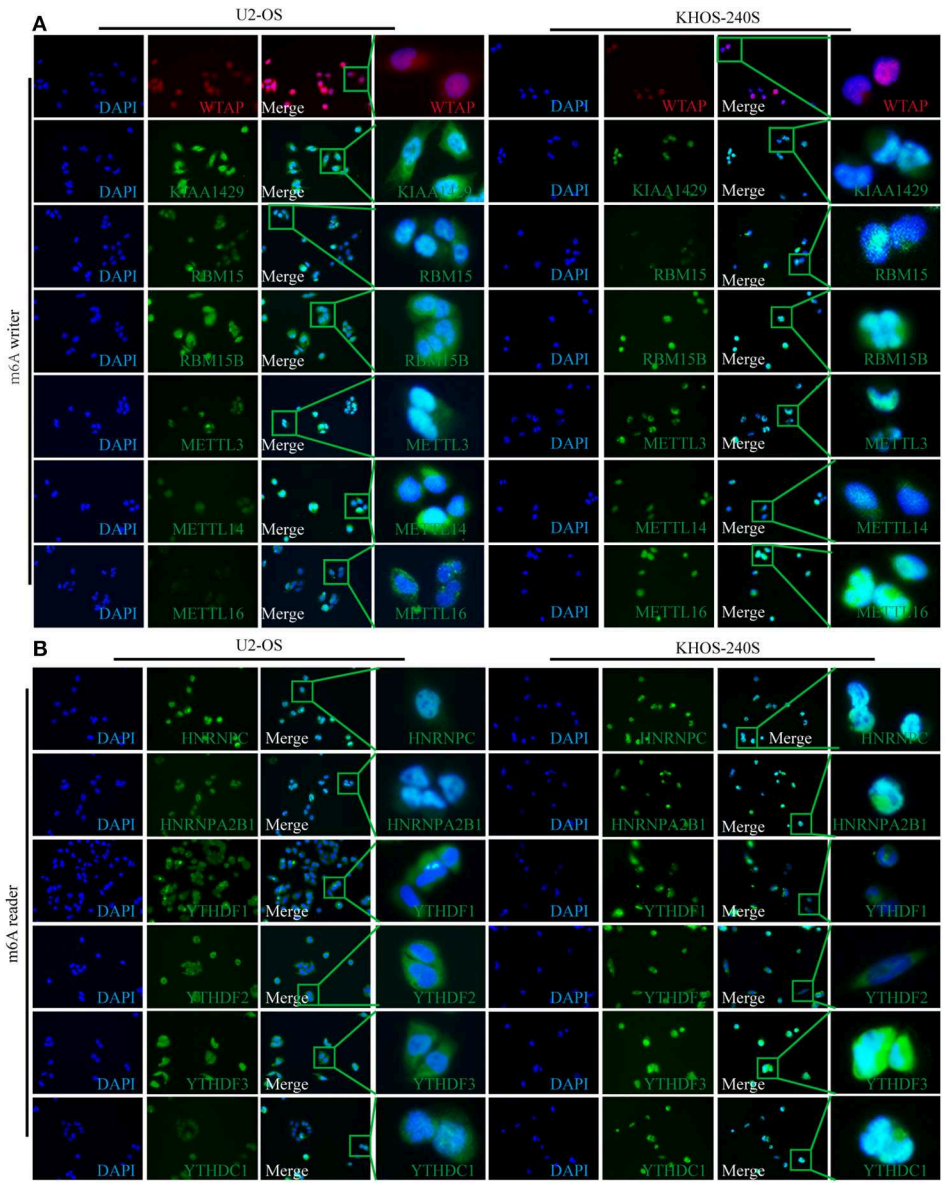


FIGURE 2 | Subcellular location of m6A-related molecules in two osteosarcoma cell lines. **(A)** Immunofluorescence of m6A "writers" in U2-OS and KHOS-240S cell lines. **(B)** Immunofluorescence of m6A "readers" in U2-OS and KHOS-240S cell lines. **(C)** Immunofluorescence of m6A "erasers" in U2-OS and KHOS-240S cell lines.

that dysregulated m6A-related regulators were associated with tumorigenesis and metastasis in OS.

Subcellular Location of m6A-Associated Proteins in Osteosarcoma Cell Lines

The diverse subcellular location of proteins may reflect on the different functions of m6A-related regulators in OS cells. Therefore, IF was performed to determine the subcellular location of m6A-related proteins in U2-OS and KHOS-240S cell lines. We found that all the m6A "writers" had intense nuclear and weak cytoplasmic staining in the two OS cell lines (**Figure 2A**). Moreover, m6A "readers" HNRNPC and HNRNPA2B1 were detected only in the nucleus, whereas YTHDF1 and YTHDF2 had weak nuclear and intense cytoplasmic staining. The fluorescence signal of YTHDF3 was intense in the nuclear and cytoplasm while the staining intensity of YTHDC1 was weak in the cytoplasm and nucleus (**Figure 2B**). As m6A methylation erasers, FTO and ALKBH5 were moderately expressed in the cytoplasm and nucleus (**Figure 2C**). The details of the subcellular localization of the m6A-related proteins in OS cells were presented in **Table S3**.

Dysregulated m6A-Related Regulators Are Associated With Poor Prognosis in Osteosarcoma

To investigate the association between m6A-related protein expression and the clinical outcome of OS patients, a TMA

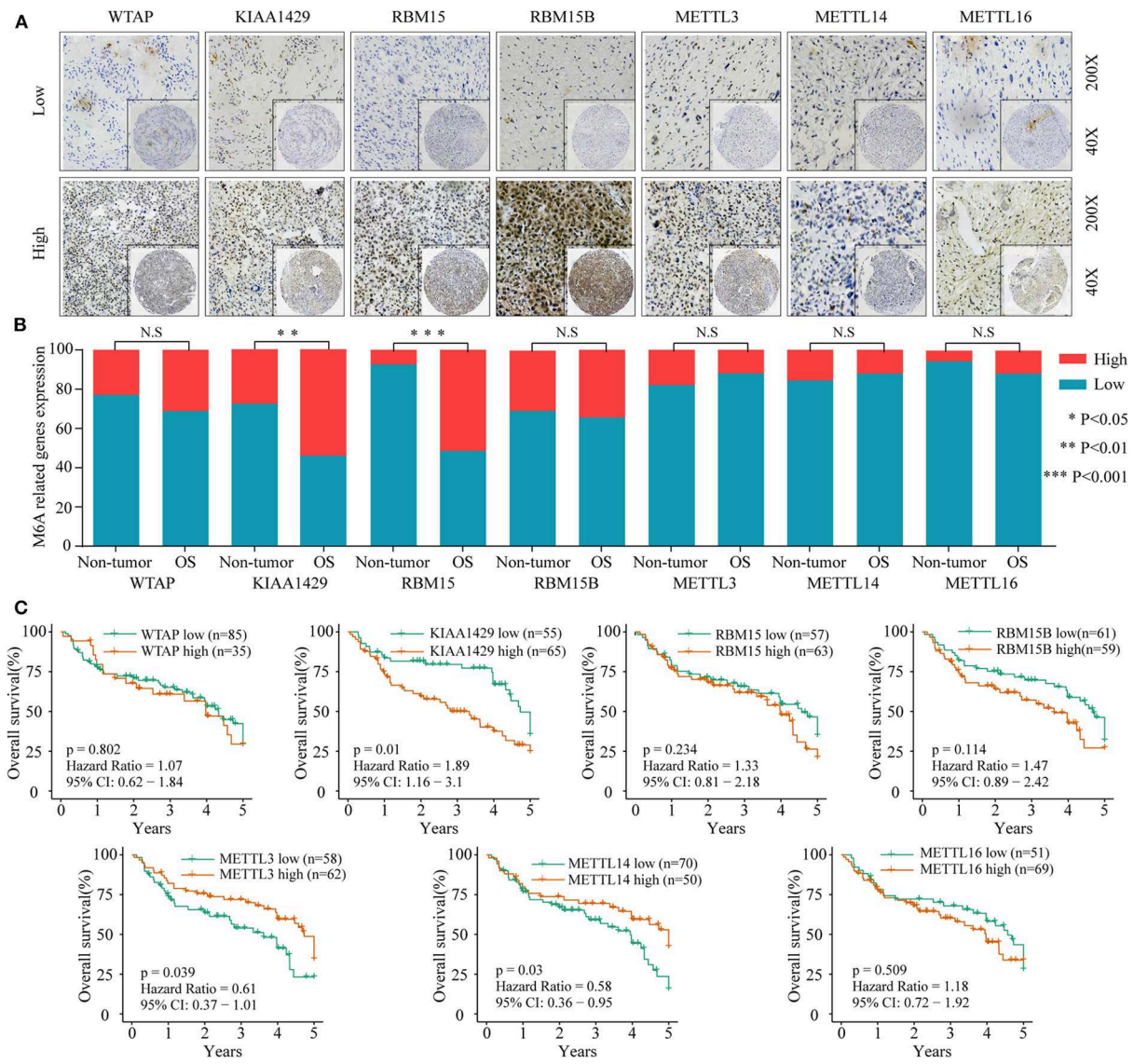


FIGURE 3 | The relationship between the expression level of m6A-related "writers" and poor prognosis in osteosarcoma. **(A)** Representative IHC high and low staining of m6A "writers" in OS tissues. **(B)** Comparison of m6A-related "writers" relative expression between OS tissues and nontumor tissues in TMA-combined cohort. **(C)** Kaplan–Meier analysis of the correlation between m6A "writers" expression and overall survival of OS patients in the TMA-combined cohort.

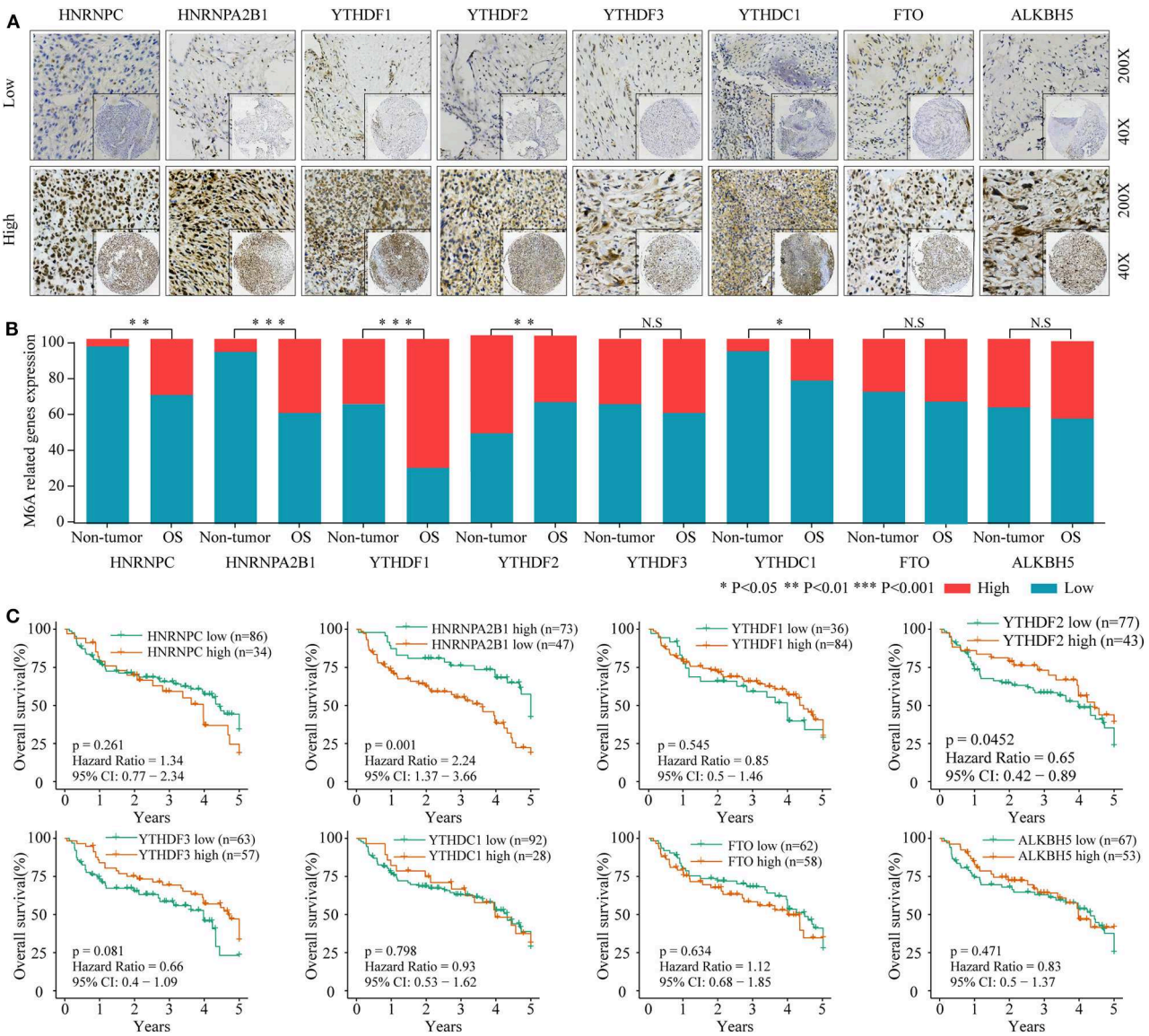


FIGURE 4 | The relationship between the expression level of m6A related "reader" and "erasers" and poor prognosis in osteosarcoma. **(A)** Representative IHC high and low staining of m6A "reader" and "erasers" in OS tissues. **(B)** Comparison of m6A "reader" and "erasers" relative expression between OS tissues and non-tumor tissues in combined-TMA cohort. **(C)** Kaplan–Meier analysis of the correlation between m6A "reader" and "erasers" expression and overall survival of OS patients in the TMA-combined cohort.

cohort containing 120 OS tissues and 65 surrounding non-tumorous tissues was employed (**Figures 3A**, **4A**). Differential expression analysis indicated that the protein expression levels of KIAA1429, RBM15, HNRNPC, HNRNPA2B1, YTHDF1, YTHDF2, and YTHDC1 were higher in tumor samples than in non-tumor samples (**Figures 3B**, **4B**). Moreover, osteosarcoma patients were divided into two groups (high and low expression groups) based on each m6A-related protein IHC staining intensity and survival analysis was performed. The results revealed that high expression of KIAA1429 and HNRNPA2B1 was significantly associated with poor overall survival rates, while low expression of METTL3, METTL14, and YTHDF2 was related to poor prognosis in OS (**Figures 3C**, **4C**).

Furthermore, an OS genome meta-cohort containing 175 OS tissues was constructed based on three independent datasets (GSE21257, GSE39055, and TARGET-OS, **Figure S1**). Overall survival analysis was performed, and the results indicated that the patients with high expression of METTL3, HNRNPA2B1, YTHDF3, and FTO and low expression of METTL14 had a significant shorter OS rate (**Figures 5A–C**). Furthermore, univariate analysis based on the TMA cohort suggested that the high expression of KIAA1429 and HNRNPA2B1 and the low expression of METTL3 and METTL14 were potential

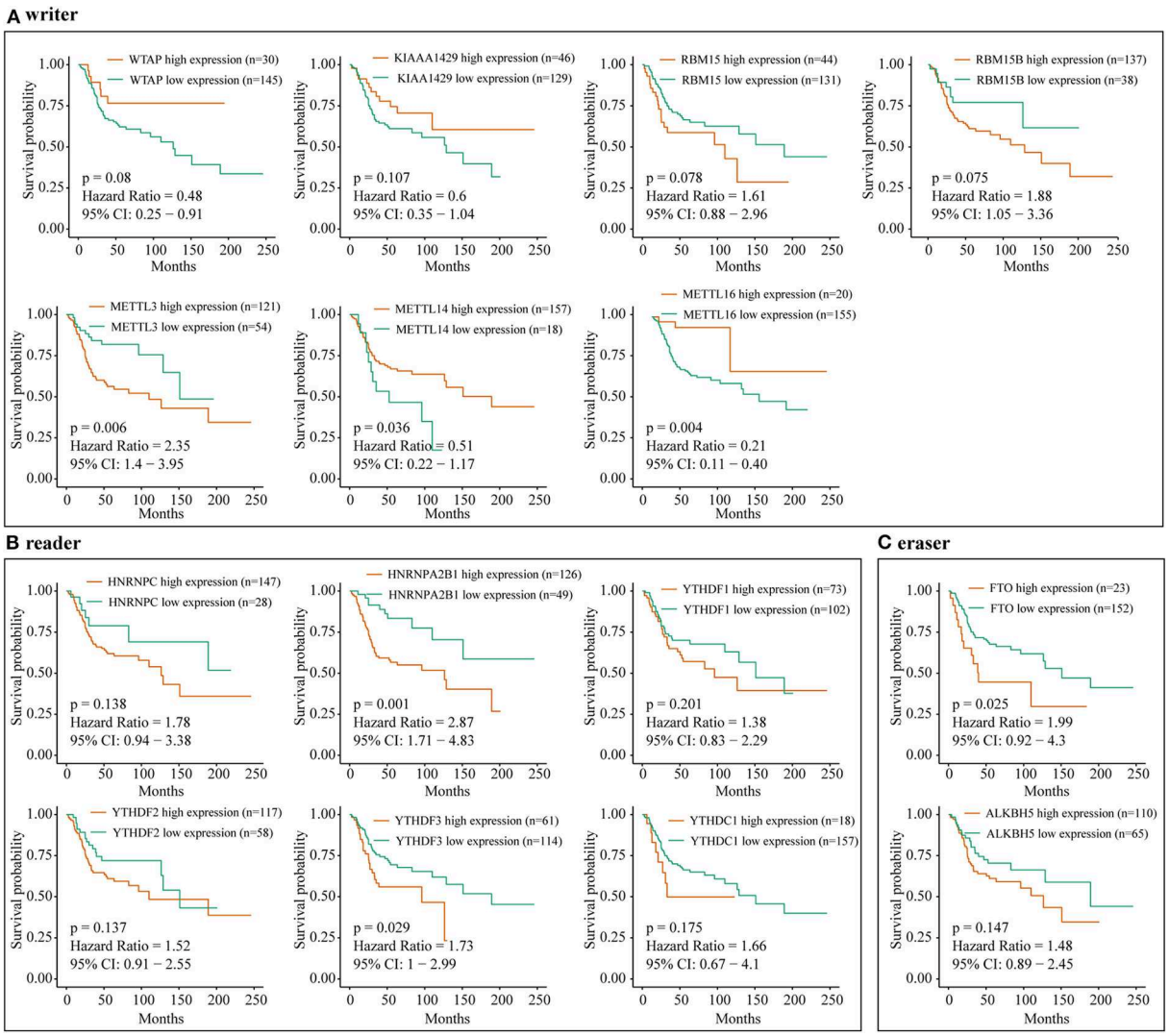


FIGURE 5 | The correlation between m6A-related regulator expression and overall survival of osteosarcoma patients in the meta-cohort. **(A)** Kaplan–Meier analysis showing the correlation between m6A "writers" expression levels and the OS patients' survival rates. **(B)** Kaplan–Meier analysis showing the correlation between m6A "readers" expression levels and the OS patients' survival rates. **(C)** Kaplan–Meier analysis showing the correlation between m6A "erasers" expression levels and the OS patients' survival rates.

independent prognostic risk factors for OS patients. Moreover, the prognostic value of HNRNPA2B1 was validated in the genome meta-cohort, and KIAA1429, METTL3, and METTL14 did not exhibit significant prognostic potential (**Figures 6A,B**). In conclusion, dysregulated m6A-related regulators may predict poor prognosis in OS.

A Cluster Associated With a Favorable Prognosis Was Identified Through Consensus Clustering of m6A-Related Genes

To further explore the prognostic value of m6A-related gene expression patterns and to understand their underlying mechanisms in OS, we analyzed the relationships among 15 m6A-related regulators. Then, we identified a cluster associated with favorable outcomes and explored its possible mechanisms based on the genome meta-cohort.

Considering the similarity of the biological functions of m6A-related regulators, we analyzed the correlation (**Figure 7A**) and interaction (**Figure 7B**) among the 15 m6A-related genes. The results indicated that there were higher correlations among HNRNPA2B1, YTHDF2, RBM15, and ALKBH5. However, there was an inverse correlation between METTL16 and HNRNPA2B1, YTHDF2, and RBM15 expression in OS. Moreover, the protein–protein interaction networks showed that these m6A-related proteins interacted with each other frequently (**Figure 7B**). Additionally, the interaction between m6A-related proteins and 150 clinically actionable genes was examined to understand the clinical significance of m6A-related proteins. The results showed

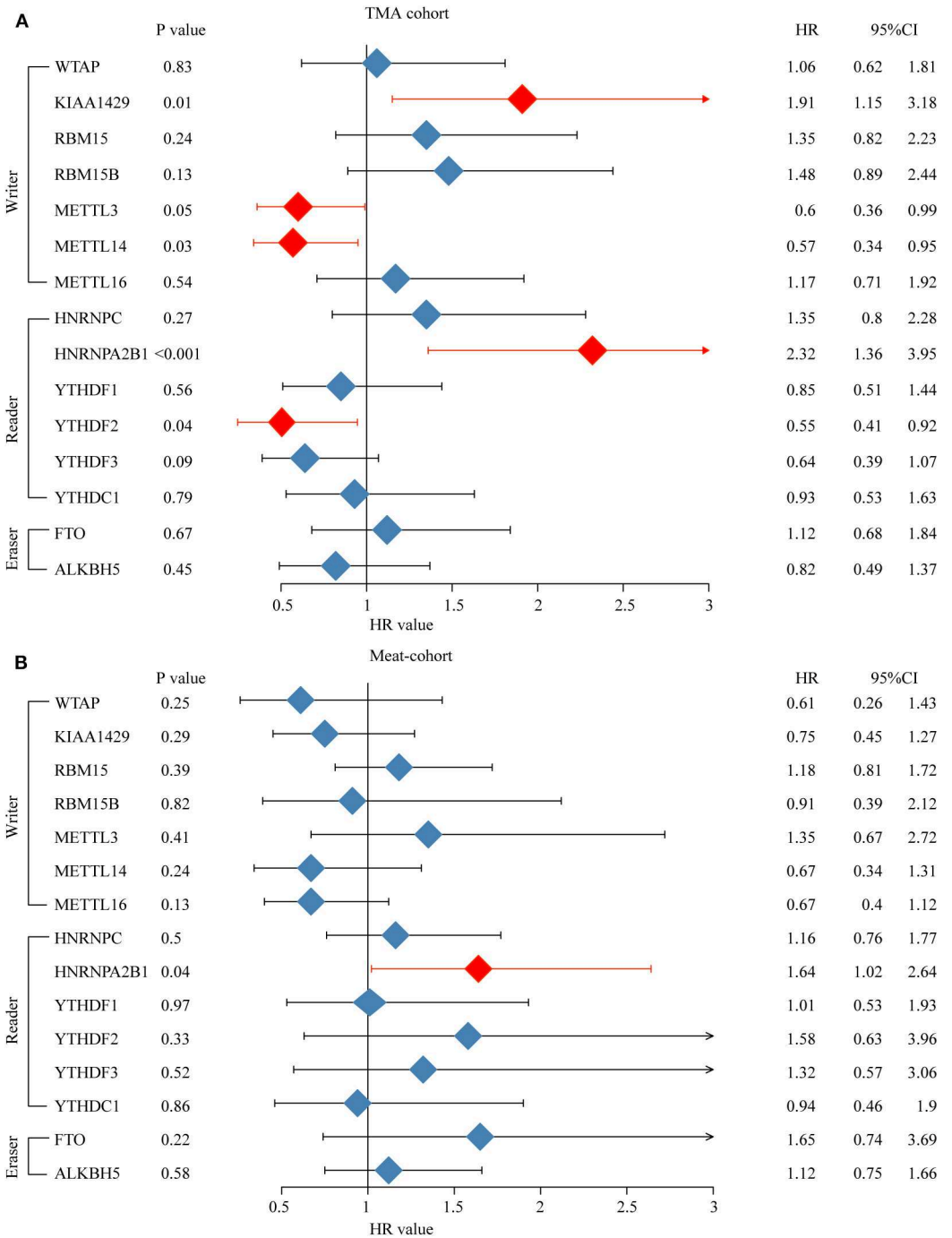


FIGURE 6 | High expression level of HNRNPA2B1 is correlated with poor prognosis of osteosarcoma. **(A)** Univariate Cox regression analysis of OS patients' overall survival in meta-cohort. **(B)** Univariate Cox regression analysis of OS patient's overall survival in TMA combined OS cohort.

that m6A-related proteins frequently interacted with some of these genes (**Figure 7B**). Conclusively, the biological functions of 15 m6A-related genes were closely related in OS.

Furthermore, three subgroups were clustered by consensus clustering based on the expression of m6A-related genes in the genome meta-cohort. Patients were divided into three clusters with K = 3. Survival analysis showed that cluster 1 had a significantly longer overall survival than cluster 2 and cluster 3 (**Figures 7C–E**). Subsequently, GSEA revealed that the humoral immune response was significantly enriched in cluster 1 which exhibited good prognosis (**Figures 7F,G**), while the cell cycle and G2/M transition of mitotic cell cycle

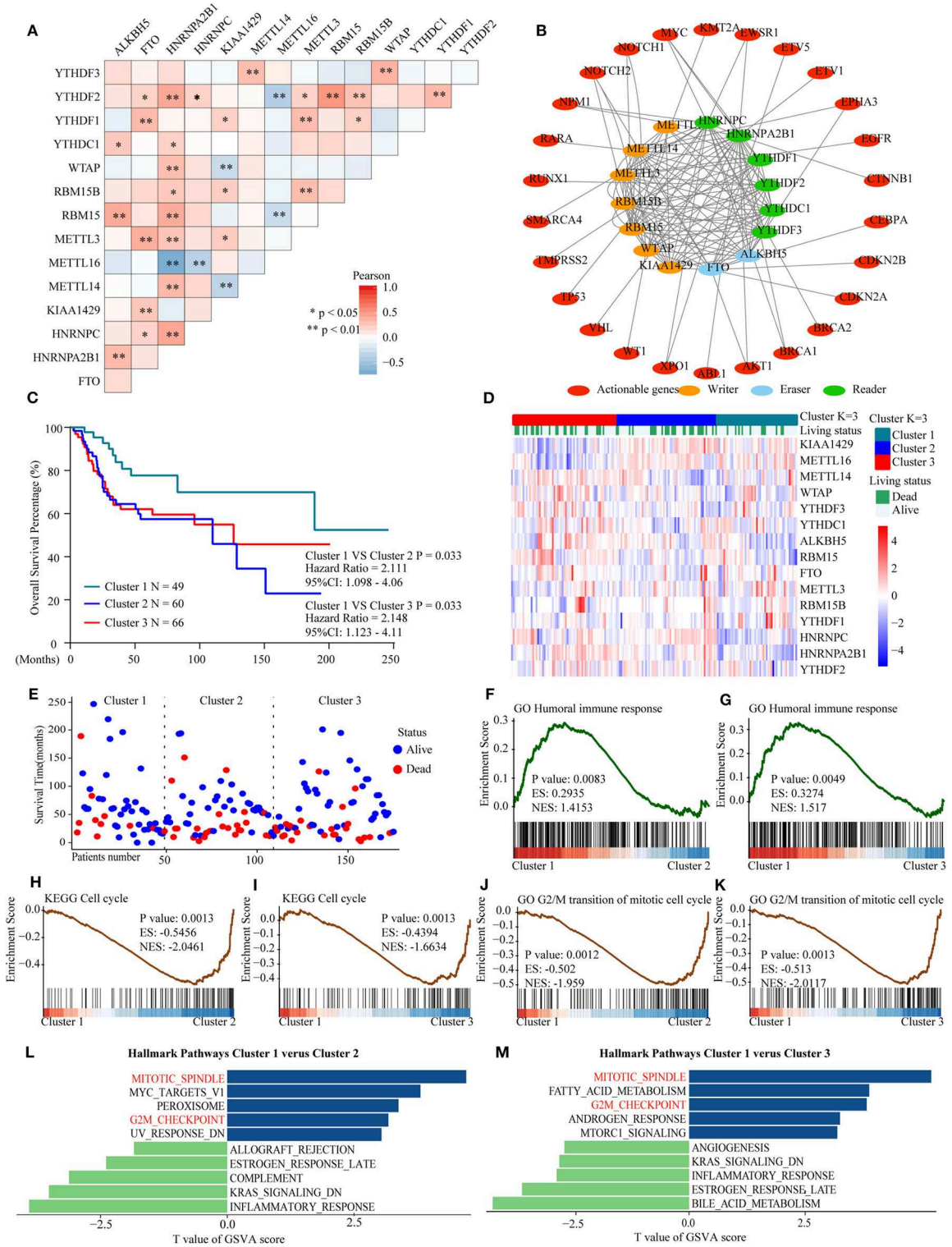


FIGURE 7 | Interaction among m6A RNA–related regulators, differential survival status, and functional annotation of OS in three clusters. **(A)** Pearson correlation analysis of the 15 m6A regulators. **(B)** Protein–protein interactions among m6A regulators and clinically actionable genes obtained from the STRING database. **(C)** Kaplan–Meier overall survival curves for 175 osteosarcoma patients. **(D)** Heatmap and survival status of the three clusters defined by the m6A related regulators' consensus expression. **(E)** Three-cluster scatter plot of different survival time and status. **(F,G)** GSEA revealed that genes with higher expression in the cluster 1 subgroup were enriched for humoral immune response. **(H,I)** GSEA revealed the relationship between three clusters in osteosarcoma and cell cycle pathway. **(J,K)** GSEA revealed the relationship between three clusters in osteosarcoma and transition of the mitotic cell cycle pathway. **(L,M)** GSVA among three clusters using hallmark gene sets.

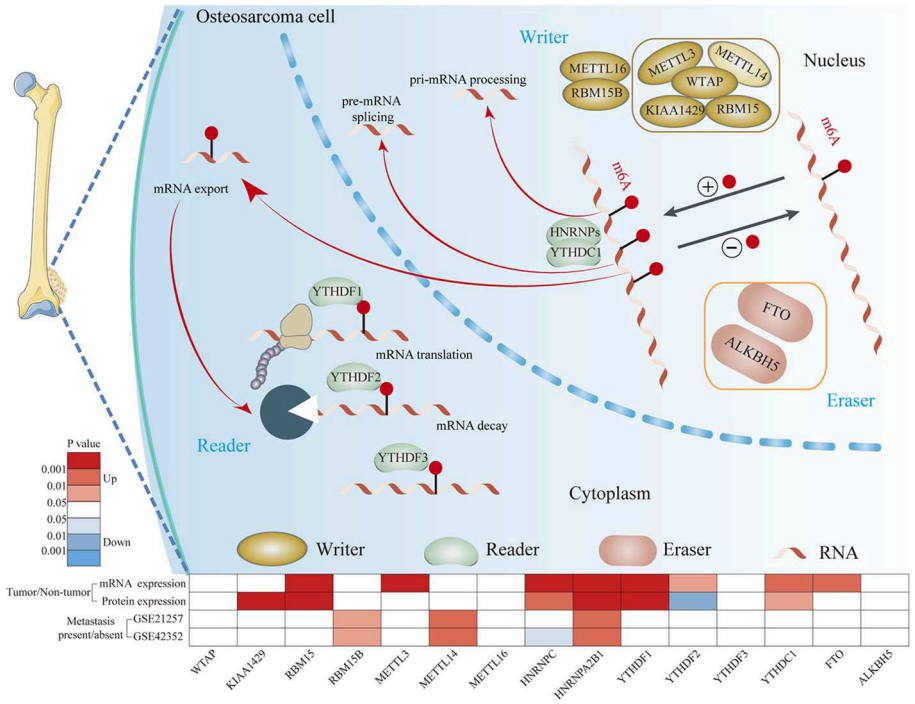


FIGURE 8 | The mechanism of m6A modification in osteosarcoma cells. m6A of RNA methylation is dynamically regulated by binding proteins "writers" (WTAP, KIAA1429, RBM15, RBM15B, METTL3, METTL14, and METTL16), "readers" (YTHDF1, YTHDF2, YTHFDF3, YTHDC1, and HNRNPs) and "erasers" (FTO and ALKBH5). "writers" add m6A modification on RNA, "readers" are proteins that bind to m6A modifications and exert various functions including pre-mRNA splicing, pri-miRNA processing, nuclear export, RNA translation modulation and mRNA decay. "erasers" could serve as demethylases.

pathways were significantly enriched in cluster 2/3 which exhibited poor prognosis (**Figures 7H–K**). Similarly, the results of GSVA indicated that the dysregulated m6A-related genes might promote mitotic spindle and G2M checkpoint pathways, thus resulting in poor prognosis in OS (**Figures 7L,M**). Overall, these findings indicated that m6A modification may be involved in OS progression by regulating the humoral immune response and cell cycle–related pathways in OS.

DISCUSSION

Many studies have demonstrated that dysregulated m6A modification is associated with human diseases such as obesity, type 2 diabetes mellitus, and infertility (27–29). In addition, the dysregulated expression of m6A-related genes is closely related to multiple tumors, including OS. To date, there have been only two reports addressing the functional role of m6A in osteosarcoma. Wang et al. demonstrated that METTL3 and ALKBH5 were closely associated with doxorubicin resistance in OS (30). Miao et al. reported that upregulated METTL3 promoted osteosarcoma progression by regulating LEF1 (31). However, the expression status and functional role of m6A-related genes in tumorigenesis and progression of OS have not yet been systematically analyzed.

In this study, we comprehensively analyzed the mRNA and protein expression levels of m6A-related regulators between OS and normal tissues based on two relatively large-scale OS cohorts (**Figure 8**). Both cohorts confirmed that the expression levels of RBM15, HNRNPC, HNRNPA2B1, YTHDF1, and YTHDC1 were increased in OS tissues. Consistent with our results, previous studies have demonstrated that RBM15 is upregulated in chronic myelogenous leukemia (32). HNRNPC and HNRNPA2B1 are upregulated in breast cancer (33, 34). YTHDF1 is upregulated in ovarian cancer, lung cancer, hepatocellular carcinoma, and colorectal cancer (35–38). YTHDC1 expression is increased in Kaposi's Sarcoma (39). In general, these findings suggest that m6A-related regulators are frequently dysregulated in OS. These dysregulated m6A-related regulators may be involved in the tumorigenesis and progression of OS.

Numerous studies have reported that dysregulated m6A-related molecule expression is closely associated with the outcome of tumor patients (23). Nevertheless, the impact of m6A methylation on OS prognosis is still unclear. For the first time, we systematically analyzed the prognostic value of m6A-related regulators in OS at both on the mRNA and protein levels. We found that high expression of HNRNPA2B1 and low expression of METTL14 were significantly associated with poor overall survival in the genome meta-cohort. Similarly, upregulated HNRNPA2B1 and downregulated METTL4 were positively related to low survival rates in the TMA cohort. Consistent with our results, HNRNPA2B1 has been reported to act as an oncogene in breast cancer, pancreatic cancer, lung cancer, hepatocellular carcinoma, and cervical cancer (34, 40–43), while METTL14 functions as a suppressor gene in glioma and breast cancer (44, 45). In contrast, the potential role of

METTL14 as an oncogene has also been observed in acute myelocytic leukemia and hepatocellular carcinoma (44, 46). In summary, the expression pattern of m6A-related regulators may be considered a potential prognostic biomarker in OS.

M6A-related regulators can play a crucial role in cancer progression by targeting downstream molecules in an m6A-dependent manner. For instance, METTL3 promotes tumor progression by regulating LEF1 and SOCS2 (13, 31); ALKBH5 promotes tumor cell proliferation by increasing FOXM1 (47); YTHDF1 silences the drug-resistance gene AKR1C1 (36); and KIAAL429 targets and downregulates GATA3, which further contributes to liver cancer progression (48). To explore the underlying mechanism involved in m6A modification–mediated OS progression, we performed a systematic bioinformatic analysis based on an OS genome meta-cohort. Frequent cross talk among m6A-related regulators was observed in OS. Subsequently, we identified three clusters by consensus clustering based on the expression of m6A-related genes and found that cluster 1 had a significantly better prognosis than the others. Bioinformatic analysis revealed that cluster 1 was closely associated with the activated humoral immune response and suppressed cell cycle–related pathways. Aberrant regulation of the immune response and cell cycle–related pathways is considered a primary causative node in cancer progression (49, 50). Interestingly, recent literature has suggested that m6A modification can regulate the cell cycle and humoral immune response pathways (51, 52). It is therefore not surprising that the potential regulatory network between m6A modification and immune response or cell cycle–related pathways, which need further investigation.

However, our present study has some shortcomings. First, we only analyzed the relationship between dysregulated m6A-related regulators and clinical features in OS but did not verify these findings through *in vivo* and *in vitro* experiments. Second, we identified that cluster 1 was associated with a better prognosis. However, these findings need further validation in more independent cohorts. Third, we only explored the underlying mechanism based on bioinformatic prediction. M6A modification–mediated aberrant activation or suppression pathways in OS remains a subject for further study.

CONCLUSION

In summary, we have identified for the first time that m6A-related regulators are frequently dysregulated and closely correlated with poor survival in OS. M6A modification–mediated aberrant activation of cell-cycle related pathways and suppression of immune response may be responsible for the crucial role of m6A in OS progression. These findings may present a promising diagnostic biomarker and a potential target therapeutic strategy for OS patients.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GEO http://www.ncbi.nlm.nih.gov/geo/, TARGET /https://ocg.cancer.gov/programs/target.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, Zhengzhou Central Affiliated Hospital of Zhengzhou University, and the Affiliated Cancer Hospital of Zhengzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL and MH performed all the experimental work. JY, CL, and HY collected osteosarcoma specimens. QH and XL participated in data analysis. RS, XC, and GC conceived and participated in the design of the study. The manuscript was written by JL and BR. All authors read and approved the final manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (81702346 and 81702757), National S&T Major Project of China (2018ZX10301201-008), National Key Research and Development Program of China (2018YFC2000500), and China Postdoctoral Science Foundation (2017M610463 and 2018M632814).

ACKNOWLEDGMENTS

We thank the many clinical doctors from the Pathology Department and Precision Medicine Center, First Affiliated Hospital of Zhengzhou University, who were involved in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00769/full#supplementary-material

REFERENCES

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. *Cancer*. (2009) 115:1531–43. doi: 10.1002/cncr.24121

2. Buddingh EP, Kuijjer ML, Duims RA, Bürger H, Agelopoulos K, Myklebost O, et al. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. *Clin Cancer Res*. (2011) 17:2110–9. doi: 10.1158/1078-0432.CCR-10-2047

3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018 CA: a cancer journal for clinicians. *ACS J.* (2018).68:7–30. doi: 10.3322/caac.21442
4. Wu Y, Zhou C, Yuan Q. Role of DNA and RNA N6-adenine methylation in regulating stem cell fate. *Curr Stem Cell Res Ther.* (2018) 13:31–8. doi: 10.2174/1574888X12666170621125457
5. Yang Y, Hsu PJ, Chen YS, Yang YG. Dynamic transcriptomic mA decoration: writers, erasers, readers and functions in RNA metabolism. *Cell Res.* (2018) 28:616–24. doi: 10.1038/s41422-018-0040-8
6. Heyn H, Esteller M. An adenine code for DNA: a second life for N6-methyladenine. *Cell.* (2015) 161:710–3. doi: 10.1016/j.cell.2015.04.021
7. Zhao BS, Roundtree IA, He C. Post-transcriptional gene regulation by mRNA modifications. *Nat Rev Mol Cell Biol.* (2017) 18:31–42. doi: 10.1038/nrm.2016.132
8. Lence T, A khtar J, Bayer M, Schmid K, Spindler L, Ho CH, et al. mA modulates neuronal functions and sex determination in *Drosophila. Nature.* (2016) 540:242–7. doi: 10.1038/nature20568
9. Du Y, Hou G, Zhang H, Dou J, He J, Guo Y, et al. SUMOylation of the m6A-RNA methyltransferase METTL3 modulates its function. *Nucleic Acids Res.* (2018) 46:5195–208. doi: 10.1093/nar/gky156
10. Visvanathan A, Patil V, Arora A, Hegde AS, Arivazhagan A, Santosh V, et al. Essential role of METTL3-mediated mA modification in glioma stem-like cells maintenance and radioresistance. *Oncogene.* (2018) 37:522–33. doi: 10.1038/onc.2017.351
11. Sadikovic B, Yoshimoto M, Chilton-MacNeill S, Thorner P, Squire JA, Zielenska M. Identification of interactive networks of gene expression associated with osteosarcoma oncogenesis by integrated molecular profiling. *Hum Mol Genet.* (2009) 18:1962–75. doi: 10.1093/hmg/ddp117
12. Liu L, Liu X, Dong Z, Li J, Yu Y, Chen X, et al. N6-methyladenosine-related genomic targets are altered in breast cancer tissue and associated with poor survival. *J Cancer.* (2019) 10:5447–59. doi: 10.7150/jca.35053
13. Chen M, Wei L, Law CT, Tsang FH, Shen J, Cheng CL, et al. RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2. *Hepatology.* (2018) 67:2254–70. doi: 10.1002/hep.29683
14. Cui X, Wang Z, Li J, Zhu J, Ren Z, Zhang D, et al. Cross talk between RNA N6-methyladenosine methyltransferase-like 3 and miR-186 regulates hepatoblastoma progression through Wnt/β-catenin signalling pathway. *Cell Prolif.* (2020) 53:e12768. doi: 10.1111/cpr.12768
15. Liu X, Liu L, Dong Z, Li J, Yu Y, Chen X, et al. Expression patterns and prognostic value of m^6A-related genes in colorectal cancer. *Am J Transl Res.* (2019) 11:3972–91.
16. Kelly AD, Haibe-Kains B, Janeway KA, Hill KE, Howe E, Goldsmith J, et al. MicroRNA paraffin-based studies in osteosarcoma reveal reproducible independent prognostic profiles at 14q32. *Genome Med.* (2013) 5:2. doi: 10.1186/gm406
17. Kuijjer ML, van den Akker BE, Hilhorst R, Mommersteeg M, Buddingh EP, Serra M, et al. Kinome and mRNA expression profiling of high-grade osteosarcoma cell lines implies Akt signaling as possible target for therapy. *BMC Med Genomics.* (2014) 7:4. doi: 10.1186/1755-8794-7-4
18. Li J, Liu L, Liu X, Xu P, Hu Q, Yu Y. DDX11 the role of upregulated as a potential prognostic and diagnostic biomarker in lung adenocarcinoma. *J Cancer.* (2019) 10:4208–16. doi: 10.7150/jca.33457
19. He Y, Chen X, Yu Y, Li J, Hu Q, Xue C, et al. LDHA is a direct target of miR-30d-5p and contributes to aggressive progression of gallbladder carcinoma. *Mol Carcinog.* (2018) 57:772–83. doi: 10.1002/mc.22799
20. Chen J, Yu Y, Li H, Hu Q, Chen X, He Y, et al. Long non-coding RNA PVT1 promotes tumor progression by regulating the miR-143/HK2 axis in gallbladder cancer. *Mol Cancer.* (2019) 18:33. doi: 10.1186/s12943-019-0947-9
21. De Winter J. Using the student's t-test with extremely small sample sizes. *Prac Assess Res Eval.* (2013) 18:12. doi: 10.7275/e4r6-dj05
22. Wilkerson MD, Hayes DN. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. *Bioinformatics.* (2010) 26:1572–3. doi: 10.1093/bioinformatics/btq170
23. Li Y, Xiao J, Bai J, Tian Y, Qu Y, Chen X, et al. Molecular characterization and clinical relevance of mA regulators across 33 cancer types. *Mol Cancer.* (2019) 18:137. doi: 10.1186/s12943-019-1066-3
24. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* (2015) 43:D447–52. doi: 10.1093/nar/gku1003
25. Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinform.* (2013) 14:7. doi: 10.1186/1471-2105-14-7
26. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS.* (2012) 16:284–7. doi: 10.1089/omi.2011.0118
27. Deng X, Su R, Stanford S, Chen J. Critical enzymatic functions of FTO in obesity and cancer. *Front Endocrinol.* (2018) 9:396. doi: 10.3389/fendo.2018.00396
28. Ding C, Zou Q, Ding J, Ling M, Wang W, Li H, et al. Increased N6-methyladenosine causes infertility is associated with FTO expression. *J Cell Physiol.* (2018) 233:7055–66. doi: 10.1002/jcp.26507
29. Klungland A, Dahl JA. Dynamic RNA modifications in disease. *Curr Opin Genet Dev.* (2014) 26:47–52. doi: 10.1016/j.gde.2014.05.006
30. Wang Y, Zeng L, Liang C, Zan R, Ji W, Zhang Z, et al. Integrated analysis of transcriptome-wide mA methylome of osteosarcoma stem cells enriched by chemotherapy. *Epigenomics.* (2019) 11:1693–715. doi: 10.2217/epi-2019-0262
31. Miao W, Chen J, Jia L, Ma J, Song D. The m6A methyltransferase METTL3 promotes osteosarcoma progression by regulating the m6A level of LEF1. *Biochem Biophys Res Commun.* (2019) 516:719–25. doi: 10.1016/j.bbrc.2019.06.128
32. Yang Y, Wang S, Zhang Y, Zhu X. Biological effects of decreasing RBM15 on chronic myelogenous leukemia cells. *Leuk Lymphoma.* (2012) 53:2237–44. doi: 10.3109/10428194.2012.684350
33. Wu Y, Zhao W, Liu Y, Tan X, Li X, Zou Q, et al. Function of HNRNPC in breast cancer cells by controlling the dsRNA-induced interferon response. *EMBO J.* (2018) 37:e99017. doi: 10.15252/embj.201899017
34. Hu Y, Sun Z, Deng J, Hu B, Yan W, Wei H, et al. Splicing factor hnRNPA2B1 contributes to tumorigenic potential of breast cancer cells through STAT3 and ERK1/2 signaling pathway. *Tumour Biol.* (2017) 39:1010428317694318. doi: 10.1177/1010428317694318
35. Liu T, Wei Q, Jin J, Luo Q, Liu Y, Yang Y, et al. The m6A reader YTHDF1 promotes ovarian cancer progression via augmenting EIF3C translation. *Nucleic Acids Res.* (2020) 48:3816–31. doi: 10.1093/nar/gkaa048
36. Shi Y, Fan S, Wu M, Zuo Z, Li X, Jiang L, et al. YTHDF1 links hypoxia adaptation and non-small cell lung cancer progression. *Nat Commun.* (2019) 10:4892. doi: 10.1038/s41467-019-12801-6
37. Zhao X, Chen Y, Mao Q, Jiang X, Jiang W, Chen J, et al. Overexpression of YTHDF1 is associated with poor prognosis in patients with hepatocellular carcinoma. *Cancer Biomark.* (2018) 21:859–68. doi: 10.3233/CBM-170791
38. Bai Y, Yang C, Wu R, Huang L, Song S, Li W, et al. YTHDF1 regulates tumorigenicity and cancer stem cell-like activity in human colorectal carcinoma. *Front Oncol.* (2019) 9:332. doi: 10.3389/fonc.2019.00332
39. Ye F, Chen ER, Nilsen TW. Kaposi's sarcoma-associated herpesvirus utilizes and manipulates RNA N-adenosine methylation to promote lytic replication. *J Virol.* (2017) 91:e00466-17. doi: 10.1128/JVI.00466-17
40. Dai S, Zhang J, Huang S, Lou B, Fang B, Ye T, et al. HNRNPA2B1 regulates the epithelial-mesenchymal transition in pancreatic cancer cells through the ERK/snail signalling pathway. *Cancer Cell Int.* (2017) 17:12. doi: 10.1186/s12935-016-0368-4
41. Dowling P, Pollard D, Larkin A, Henry M, Meleady P, Gately K, et al. Abnormal levels of heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) in tumour tissue and blood samples from patients diagnosed with lung cancer. *Mol Biosyst.* (2015) 11:743–52. doi: 10.1039/C4MB00384E
42. Wang H, Liang L, Dong Q, Huan L, He J, Li B, et al. Long noncoding RNA miR503HG, a prognostic indicator, inhibits tumor metastasis by regulating the HNRNPA2B1/NF-κB pathway in hepatocellular carcinoma. *Theranostics.* (2018) 8:2814–29. doi: 10.7150/thno.23012
43. Shi X, Ran L, Liu Y, Zhong SH, Zhou PP, Liao MX, et al. Knockdown of hnRNP A2/B1 inhibits cell proliferation, invasion and cell cycle triggering apoptosis in cervical cancer via PI3K/AKT signaling pathway. *Oncol Rep.* (2018) 39:939–50. doi: 10.3892/or.2018.6195
44. Wang S, Sun C, Li J, Zhang E, Ma Z, Xu W, et al. Roles of RNA methylation by means of N-methyladenosine (mA) in human cancers. *Cancer Lett.* (2017) 408:112–20. doi: 10.1016/j.canlet.2017.08.030

45. Wu L, Wu D, Ning J, Liu W, Zhang D. Changes of N6-methyladenosine modulators promote breast cancer progression. *BMC Cancer.* (2019) 19:326. doi: 10.1186/s12885-019-5538-z
46. Weng H, Huang H, Wu H, Qin X, Zhao BS, Dong L, et al. METTL14 inhibits hematopoietic stem/progenitor differentiation and promotes leukemogenesis via mRNA mA modification. *Cell Stem Cell.* (2018) 22:191–205.e9. doi: 10.1016/j.stem.2017.11.016
47. Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, et al. mA demethylase ALKBH5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. *Cancer Cell.* (2017) 31:591–606.e6. doi: 10.1016/j.ccell.2017.02.013
48. Lan T, Li H, Zhang D, Xu L, Liu H, Hao X, et al. KIAA1429 contributes to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of GATA3. *Mol Cancer.* (2019) 18:186. doi: 10.1186/s12943-019-1106-z
49. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature.* (2014) 515:563–7. doi: 10.1038/nature14011
50. Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. *Nat Rev Cancer.* (2017) 17:93–115. doi: 10.1038/nrc.2016.138
51. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. *Nat Rev Cancer.* (2014) 14:722–35. doi: 10.1038/nrc3838
52. Williams SA, Maecker HL, French DM, Liu J, Gregg A, Silverstein LB, et al. USP1 deubiquitinates ID proteins to preserve a mesenchymal stem cell program in osteosarcoma. *Cell.* (2011) 146:918–30. doi: 10.1016/j.cell.2011.07.040

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 14 December 2020
doi: 10.3389/fonc.2020.581107

Targeting Tumor-Associated Macrophages in the Pediatric Sarcoma Tumor Microenvironment

*Jane Koo, Masanori Hayashi, Michael R. Verneris and Alisa B. Lee-Sherick**

Department of Pediatric Hematology/Oncology/Bone Marrow Transplant, University of Colorado School of Medicine, Children's Hospital Colorado, Aurora, CO, United States

For many pediatric sarcoma patients, multi-modal therapy including chemotherapy, radiation, and surgery is sufficient to cure their disease. However, event-free and overall survival rates for patients with more advanced disease are grim, necessitating the development of novel therapeutic approaches. Within many pediatric sarcomas, the normal immune response, including recognition and destruction of cancer cells, is lost due to the highly immune suppressive tumor microenvironment (TME). In this setting, tumor cells evade immune detection and capitalize on the immune suppressed microenvironment, leading to unchecked proliferation and metastasis. Recent preclinical and clinical approaches are aimed at understanding this immune suppressive microenvironment and employing cancer immunotherapy in an attempt to overcome this, by renewing the ability of the immune system to recognize and destroy cancer cells. While there are several factors that drive the attenuation of immune responses in the sarcoma TME, one of the most remarkable are tumor associated macrophage (TAMs). TAMs suppress immune cytolytic function, promote tumor growth and metastases, and are generally associated with a poor prognosis in most pediatric sarcoma subtypes. In this review, we summarize the mechanisms underlying TAM-facilitated immune evasion and tumorigenesis and discuss the potential therapeutic application of TAM-focused drugs in the treatment of pediatric sarcomas.

Keywords: pediatric sarcoma, tumor-associated macrophage, efferocytosis, tumor microenvironment, immunotherapy

OPEN ACCESS

Edited by:
Alan Wayne,
Children's Hospital of Los Angeles, United States

Reviewed by:
Rosandra Natasha Kaplan, National Cancer Institute (NCI), United States
Shahab Asgharzadeh, University of Southern California, United States

***Correspondence:**
Alisa B. Lee-Sherick
alisa.leesherick@cuanschutz.edu

Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *07 July 2020*
Accepted: *09 November 2020*
Published: *14 December 2020*

Citation:
Koo J, Hayashi M, Verneris MR and Lee-Sherick AB (2020) Targeting Tumor-Associated Macrophages in the Pediatric Sarcoma Tumor Microenvironment. Front. Oncol. 10:581107. doi: 10.3389/fonc.2020.581107

INTRODUCTION

Pediatric sarcomas are a heterogenous group of tumors that comprise approximately 10% of all childhood cancers (1–5). While sarcomas also occur in adults, the prevalence of subtypes is strikingly unique for the pediatric population. The most common bony pediatric sarcomas are osteosarcoma and Ewing sarcoma (EWS), while rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma. Other rarer sarcoma subtypes such as synovial sarcoma, leiomyosarcoma, and liposarcomas can occur in children, but are more common in adult patients (6). The cornerstone of treatment typically involves an intensive multi-modality

approach including cytotoxic chemotherapy, surgery, and radiation. Over the last five decades, survival improvements have resulted from incremental adjustments to current therapy; however, very few new therapies have been shown to positively improve pediatric sarcomas outcomes (7–9).

For patients with metastatic RMS, the 3-year overall survival (OS) and event-free survival (EFS) are 34 and 27% respectively (10, 11). Survival rates for metastatic osteosarcoma and EWS are similarly dismal with 5-year survival rates reported between 20–30% and 30–40%, respectively (10, 11). Current therapies are highly toxic and associated with many short- and long-term side effects resulting in considerable life-long morbidities (12–15). Alternative approaches, such as immunotherapy, are desperately needed to both improve cure rates and to minimize long-term side effects. Therapeutic approaches that direct the immune system to recognize and destroy tumor cells are currently being trialed in patients with relapsed/refractory sarcomas.

To better understand the potential benefit of immunotherapy in pediatric sarcomas, certain biologic and mutational differences between pediatric and adult sarcomas warrant emphasis. In contrast to adult sarcomas, pediatric sarcomas are generally characterized by a low mutational burden, specific chromosomal translocations that encode "driver mutations," and low somatic copy number alterations in some sarcoma subtypes (16–23). Higher mutational burden and presence of complex genomic aberrations that occur in adult patients may increase the presence and immune recognition of sarcoma neoantigens. This is further compounded by the observation that the pediatric adaptive immune system tends to be more plastic and may account for variations in individual responses to immunotherapy (24, 25). Additionally, there is higher marrow cellularity and more robust hematopoiesis in children compared to adult patients, exemplified by faster immune reconstitution in children following chemotherapy (26–28). Therefore, these unique differences in sarcoma biology and immune function between adult and pediatric patients likely affect responses to immunotherapy. Furthermore, before cellular immunotherapy can be fully leveraged for pediatric sarcomas an understanding of TAMs within the sarcoma TME is required.

THE SARCOMA TUMOR MICROENVIRONMENT

The cellular composition of the TME is broadly comprised of tumor cells, non-malignant stromal cells, blood vessels, and immune cells. Stromal cells produce extracellular matrix (ECM) proteins and matricellular proteins that provide structural support and mediate signaling for cellular movement. The immune components of the sarcoma TME, including innate immune cells [neutrophils, TAMs, natural killer (NK) cells, dendritic cells (DCs)] and adaptive immune cells (B and T lymphocytes), can vary vastly with respect to sarcoma subtype, primary tumor location, genetic or mutational burden and previous therapy exposure. TAMs are one of at least four myeloid subpopulations derived from tumor-associated myeloid cells (TAMCs) that also include myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs), and angiogenic monocytes expressing angiopoietin-2 (TIE-2) (29–31). Cellular immunotherapeutic approaches have largely tested adopted transfer of activated and/or antigen specific T cells; however, efficacy of these cells can be significantly dampened by cells that exert immune regulatory function, including TAMs, regulatory T cells (Tregs), and mesenchymal stem cells (MSCs). For the purposes of this review, we focus on TAMs.

Several studies have demonstrated a strong correlation between macrophage infiltration, sarcoma tumor progression, and patient survival, highlighting TAMs as potential immunotherapeutic targets in pediatric sarcoma (32–36). In addition to phagocytosis of necrotic tumor cells, which decreases the presence of tumor antigen and subsequent immunogenic T cell response, TAMs have been shown to display a wide variety of immunosuppressive and tumor-promoting functions. For instance, increased proportion of TAMs has been shown to render chimeric antigen receptor T cell immunotherapy ineffective (37). However, TAM number and density in pediatric sarcomas do not explain the entirety of their importance in facilitating tumor progression, and the immune cell profiles in pediatric sarcomas vary across tumor subtypes.

MACROPHAGES IN TUMORIGENESIS

Macrophages play critical roles in innate immunity including: phagocytosis, clearance of apoptotic debris, lymphocyte recruitment (38, 39), antigen presentation (40, 41), wound healing (42), and tissue homeostasis (43, 44). Thus, they both promote inflammatory responses as well as facilitate resolution. In the setting of cancer, macrophages have a response that is seemingly antithetical to the whole organism, as they drive immune tolerance and facilitate cancer progression (45).

Various clinically applicable techniques are in development to identify, quantify, and characterize sarcoma-associated TAMs. Such techniques include immunohistochemistry, single cell RNA sequencing, fluorescent magnetic nanoparticle labeling, and even non-invasive imaging including magnetic resonance imaging (MRI), given that T2* signal enhancement on MR images significantly correlated with TAM density in sarcoma patients (32, 46–48). Depending on their local microenvironments, TAMs can display phenotypic and functional heterogeneity, which is best understood through the concept of macrophage polarization (see next paragraph) (49). However, this dichotomous polarization paradigm is largely oversimplified, and there is a broad range of macrophage polarization phenotypes *in vivo* (50). While TAMs are the largest population of infiltrating immune cells within pediatric sarcomas and TAM infiltration into the tumor can be linked with worse prognosis, the density of TAMs within the tumor does not necessarily provide the full scope of how they influence the TME (34, 51).

MACROPHAGE POLARIZATION IN TUMOR DEVELOPMENT

The M1/M2 polarization spectrum was developed to explain macrophage phenotype and function in response to inflammation or infection. In the setting of inflammation, M1 macrophages (classically activated macrophages) migrate to sites of infection, phagocytose infected cells and serve as antigen presenting cells (APCs) and produce T helper cell type 1 (Th1) or pro-inflammatory cytokines, promoting T cell activation. In contrast, M2 (alternatively activated) macrophages promote tissue repair through efferocytosis, a phagocytic process in which antigen are cleared, antigen presentation is diminished, and T helper cell type 2 (Th2) cytokines are produced. This process also promotes immune tolerance to autologous (or "self") tissue. Macrophage plasticity and polarization in the sarcoma TME is also critical for the progression or regression of these tumors (**Figure 1**).

Following exposure to damage- or pathogen-associated molecular patterns (DAMPs or PAMPs), such as bacterial lipopolysaccharides (LPS), nucleic acids, and other microbial ligands, toll-like receptor (TLR) are triggered and M1 polarize macrophage (52–54). TLR ligation initiates a signaling cascade involving the innate immune signal transduction adaptor MYD88, interleukin 1 receptor associated kinase 4 (IRAK4), tumor necrosis factor associated factor 6 (TRAF6) and inhibitor of nuclear factor kappa B kinase subunit beta (IKK-β) which ultimately activates nuclear factor kappa B (NF-κB), one of the central regulators of inflammatory cytokine production. Translocation of NF-κB into the nucleus leads to transcription of Th1 genes, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-12, IL-1β, and IL-6, leading to expansion of effector T cells (55–60). Activated T cells produce pro-inflammatory cytokines (*e.g.*, interferon gamma (IFN- γ), granulocyte colony-stimulating factor (GM-CSF)) further perpetuating macrophage M1 activation (61–63). Additionally, GM-CSF is a potent driver of antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), a cell mediated immune defense whereby immune effector cells destroy antibody coated target

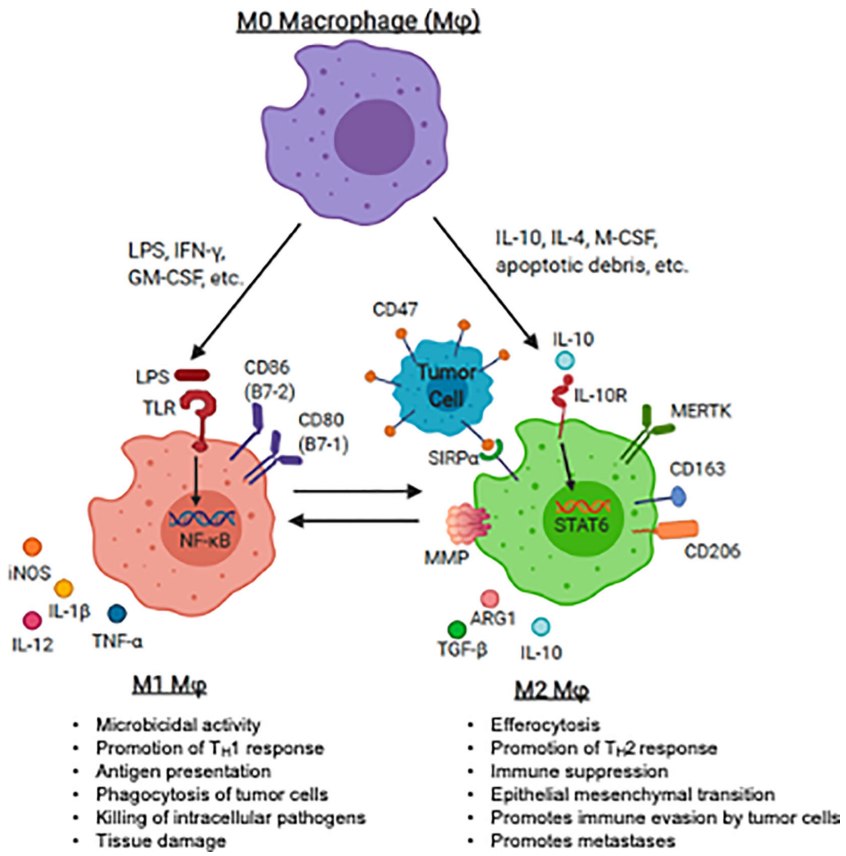


FIGURE 1 | Macrophage polarization and plasticity within the pediatric sarcoma tumor microenvironment. The panel represents recognized M1 (anti-tumoral) and M2 (tumor-promoting) agonists that induce the induction of M1 and M2 markers by human macrophages. The major canonical functions of M1 macrophages and M2 macrophages are also described. LPS, lipopolysaccharide, IFN-γ, interferon-gamma; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-4, interleukin 4; IL-10, interleukin-10; IL-13, interleukin 13; M-CSF, macrophage colony stimulating factor; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin 1 beta; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; MMP, matrix metalloprotease; MERTK, Mer receptor tyrosine kinase; TGF-β, transforming growth factor beta. Image created with biorender.com.

cells (64–66). M1 macrophages generally express high levels of surface molecules for antigen presentation (e.g., major histocompatibility complex-II (MHC-II)), augment T cell activation (*e.g.*, CD80, CD86), and further promote self-activation (*e.g.*, TLR2, TLR4) (67). M1 macrophages produce cytokines to amplify T cell activation, such as IL-1β, IL-6, IL-12, IL-18 and TNF-α (68, 69).

Alternative activation, or M2 polarization, is thought to occur after exposure to cytokines such as IL-4, IL-10, macrophage colony-stimulating factor (M-CSF) and transforming growth factor beta (TGF-β) (70, 71), and/or apoptotic cellular debris which promote the resolution of inflammation and wound-healing. M2 macrophages may be identified by the up-regulation of surface markers that promote clearance of apoptotic debris, such as mannose receptor C-type 1 (MMR, CD206) and CD163 (63, 72–76). M2 macrophages may produce T cell suppressive cytokines such as TGF-β and IL-10 (77). In response to local cytokine milieu, alternatively activated macrophages also up-regulate inhibitory checkpoint ligands, such as programmed death 1 ligand 1 (PD-L1) and programmed death 1 ligand 2 (PD-L2), which inhibit T cell effector function (78, 79). Many of the above pathways have been or are being considered for targeting to either augment immunity or inhibit the counter-regulatory activity known to occur in malignancy. A summary of therapeutic strategies targeting TAMs in the pediatric sarcoma TME is summarized in **Figure 2**.

TLR Agonists

Manipulating macrophage polarization in the TME toward M1 activation status has been evaluated using TLR agonists. Muramyl TriPeptide-PhosphatidylEthanolamine encapsulated into liposomes (L-MTP-PE) has been proposed as an adjuvant therapy for osteosarcoma patients. It is a synthetic analog of muramyl dipeptide (MD), a peptidoglycan that is found in bacterial cell walls. L-MTP-PE has been demonstrated to activate TLR4 on macrophages and monocytes and upregulate their tumoricidal functions through increased type 1 cytokine production (such as TNF-α, IL-1, IL-6, IL-8, IL-12, and nitric oxide (NO)) (80, 81). A preclinical evaluation of L-MTP-PE combined with zoledronic acid (ZA) in murine models of osteosarcoma showed that the two drugs significantly inhibited tumor growth and development of metastases (82). Phase I and II clinical trials evaluating L-MTP-PE in pediatric osteosarcoma patients showed acceptable toxicity, and even enhanced macrophage-mediated tumoricidal activity, but had variable results in prolongation of OS and EFS (see **Table 1**) (80, 90, 91). In a follow-up randomized phase III trial [Intergroup (INT)-0133] by the Children's Oncology Group (COG) for patients with osteosarcoma addition of L-MTP-PE to standard chemotherapy showed no difference in 5-year OS or EFS. When patients with metastatic disease were analyzed separately, L-MTP-PE had improved survival compared (53 *vs* 40%); however, but the study was not powered to detect a

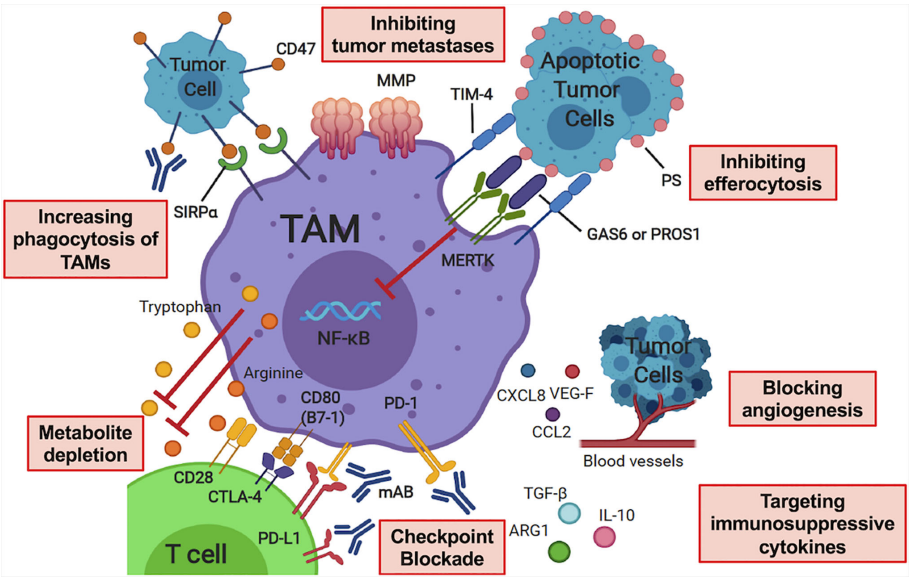


FIGURE 2 | Therapeutic Strategies Targeting Tumor-Associated Macrophages in the Pediatric Sarcoma Microenvironment. Therapy modalities include increasing phagocytosis of TAMs, inhibiting tumor metastases, inhibiting efferocytosis, checkpoint blockade, altering macrophage polarization through targeting immunosuppressive cytokines, metabolite depletion and blocking angiogenesis. TAM, tumor-associated macrophage; SIRPα, signal-regulatory protein alpha; MMP, matrix metalloprotease; PS, phosphatidylserine; TIM-4, T Cell Immunoglobulin And Mucin Domain Containing 4; MERTK, Mer receptor tyrosine kinase; PROS1, protein S; GAS6, growth arrest-specific 6; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; mAB, monoclonal antibody; IL-10, interleukin 10; TGF-β, transforming growth factor beta; ARG1, arginase 1; VEG-F, vascular endothelial growth factor; CXCL8, C-X-C motif chemokine ligand 8; CCL2, C-C motif chemokine ligand 2. Image created with biorender.com

TABLE 1 | Current macrophage targeted therapies for the treatment of pediatric sarcomas.

Class	Name	Target	Form	Studies in pediatric sarcoma	Outcomes	Reference
Cytokines						
	GM-CSF[1]	Macrophages	Inhaled Inhaled SC[6]	Phase I dose escalation studies in pediatric cancer patients AOST0221: Phase II study of inhaled GM-CSF in first pulmonary recurrence of osteosarcoma patients Phase II study of GM-CSF in combination with chemotherapy and radiation in EWS patients	Limited to no toxicity observed one patient with EWS[2] achieved a CR[3]; 3-year EFS[4] and OS[5] were 7.8 and 35.4%, respectively EFS for 96 patients with osteosarcoma 12% at 4 months; EFS for 42 evaluable patients 20% at 12 months 5-year EFS of non-metastatic EWS patients in group A (with GM-CSF); 0.56 *vs* 5-year EFS in group B (without GM-CSF 0.51. EFS for metastatic EWS was not calculated due to small numbers	(83, 84) (85) (86)
	Zoledronic Acid	Macrophages	IV[7] IV IV	AOST06P1: Phase I study of ZA[8] in metastatic OS patients Phase II study of ZA at standard dosing for metastatic osteosarcoma patients EURO-EWING 2012: Phase III randomized, multi-center study combining ZA with standard chemotherapy for EWS patients	DLTs[9] experienced by five of 24 patients. DLTs included hypophosphatemia, hypokalemia, hyponatremia, mucositis, limb pain and edema; overall EFS and OS for 24 patients were 32% and 60%, respectively Median PFS[10] 19 months; median OS 56 months among four patients Clinical trial is currently ongoing. (ISRCTN92192408)	(87) (88) (89)
	L-MTP-PE[11]	Macrophages/ Monocytes	IV IV	Phase I study of L-MTP-PE in advanced malignancies Phase IIb study of L-MTP-PE in combination with ifosfamide with relapsed osteosarcoma Intergroup-0133: Phase III randomized trial of addition of L-MTP-PE to standard chemotherapy in pediatric patients with metastatic osteosarcoma	Toxicities included fever, chills and hypertension; no major organ-related toxicities observed No increased toxic side effects observed when ifosfamide combined with L-MTP-PE 5-year EFS for patients who received L-MTP-PE *vs* no L-MTP-PE was 46 *vs* 26%, respectively. 5-year OS for patients who received L-MTP-PE vs no L-MTP-PE was 53 and 40%, respectively.	(90) (91) (92)
	Recombinant TNF	Macrophages	IV	Phase I study of rTNF[12] combined with a fixed dose of actinomycin D in pediatric patients with refractory malignancies	At 240 μg/m2/day of rTNF, three of six patients experienced grade 4 DLT including hypotension, hemorrhagic gastritis, and renal and liver biochemical alterations; antitumor response observed in one metastatic EWS patient	(93)
Checkpoint inhibitors						
	Nivolumab	PD-1[13]	IV	Phase II study of nivolumab with or without ipilimumab in patients with unresectable metastatic sarcoma	Clinical trial is currently active not recruiting (NCT02500797).	-
	Pembrolizumab	PD-1	IV IV IV	SARC028: phase II study of pembrolizumab assessing safety and activity in patients with advanced soft-tissue or bone sarcomas Phase II study of pembrolizumab and axitinib in patients with advanced alveolar soft part sarcoma and other soft tissue sarcomas PEMBROSARC: Phase II multi-center trial of pembrolizumab with metronomic cyclophosphamide administration in advanced sarcoma patients	Seven (18%) of 40 patients with soft-tissue sarcoma had an objective response two (5%); of 40 patients with bone sarcoma had an objective response including one (5%) of 22 patients with osteosarcoma and one (20%) of five patients with chondrosarcoma. None of the 13 patients with EWS had an objective response. (NCT02301039) Clinical trial is currently active, not recruiting. (NCT02636725) Clinical trial is currently active, recruiting. (NCT02406781)	(94) - -
	Ipilimumab	PD-1	IV	NCI 08-C-0007: Phase I study of ipilimumab in pediatric patients with recurrent/refractory solid tumors	Immune-related adverse events included pancreatitis, colitis, endocrinopathies and transaminitis. DLTs observed at 5 and 10 mg/kg/dose levels of ipilimumab; one osteosarcoma, one synovial sarcoma and one clear cell sarcoma patient had stable disease for 4–10 cycles. (NCT0144537)	(95)
Macrophage immunosuppression inhibitors						
	CB-1158 (INCB00158)	Arginase	IV	Open-label phase I/phase II evaluation of arginase inhibitor INCB00158 as single agent and in combination with pembrolizumab	Clinical trial is currently active, recruiting. (NCT02903914)	-

(Continued)

TABLE 1 | Continued

Class	Name	Target	Form	Studies in pediatric sarcoma	Outcomes	Reference
				for patients with advanced/ metastatic solid tumors		
Angiogenesis inhibitors						
	Bevacizumab	VEG-F	IV IV IV IV IV	Observational off-label study of bevacizumab in combination with cytotoxic chemotherapy salvage and maintenance regimens in pediatric patients with relapsed/ refractory sarcomas Phase 1 COG[14] study of bevacizumab in pediatric patients with refractory solid tumors Phase I study of bevacizumab combined with irinotecan in patients with recurrent, progressive, refractory solid tumors Phase I study of bevacizumab combined with vincristine, irinotecan and temozolomide in pediatric patients with relapsed tumors Phase I study of bevacizumab combined with sorafenib and low-dose cyclophosphamide in pediatric patients with refractory/ recurrent solid tumors	Most frequent side effects included epistaxis, transaminitis, acral dermatitis, hypertension, and albuminuria. No DLTs were observed. Non-DLTs included infusion reaction, rash, mucositis, proteinuria, and lymphopenia. DLTs included diarrhea, neutropenia/thrombocytopenia. Maximum-tolerated dose was bevacizumab 10 mg/kg and irinotecan 100 mg/m^2 DLTs included hyperbilirubinemia and colitis. Other toxicities included diarrhea, hypertension, and myelosuppression. DLTs included rash, lipase elevation, anorexia, and thrombus. Other common toxicities included neutropenia, lymphopenia and rashes.	(96) (97) (98) (99) (100)
Metastasis inhibitors						
	Pexidartinib (PLX3397)	CSF1R[15]	IV IV	Phase I/II trial of PLX3397 in pediatric patients with refractory solid tumors and leukemias Phase Ib study of pexidartinib combined with paclitaxel in patients with advanced solid tumors	Clinical trial is currently active, recruiting. (NCT02390752) Adverse events included anemia, neutropenia, fatigue, and hypertension	- (101)

[1]*GM-CSF, Granulocyte-macrophage colony stimulating factor.*
[2]*EWS, Ewing Sarcoma.*
[3]*CR, Complete response.*
[4]*EFS, Event-free survival.*
[5]*OS, Overall survival.*
[6]*SC, Subcutaneous.*
[7]*IV, Intravenous.*
[8]*ZA, Zoledronic acid.*
[9]*DLT, Dose-limiting toxicity.*
[10]*PFS, progression-free survival.*
[11]*L-MTP-PE, Liposomal-Muramyl TriPeptide-PhosphatidylEthanolamine.*
[12]*rTNF, recombinant TNF.*
[13]*PD-1, Programmed cell death 1.*
[14]*COG, Children's Oncology Group.*
[15]*CSF1R, Colony stimulating factor 1 receptor.*

significant difference between the two arms (92). L-MTP-PE is not currently approved by the United States Food and Drug Administration (FDA) (102) though the European Medicines Agency granted L-MTP-PE an indication as an adjuvant treatment of osteosarcoma in 2009.

Re-Polarizing Agents

Administration of exogenous cytokines to reverse TAM M2 polarization may be an effective immunotherapeutic strategy for pediatric sarcomas. GM-CSF is a myeloid growth factor that stimulates the differentiation of hematopoietic progenitor cells into granulocytes and monocytes with subsequent type 1 cytokine mRNA expression, such as IL-1β, IL-6 and TNF (103). GM-CSF has been successfully incorporated into the standard therapy of high-risk neuroblastoma patients receiving antibody therapy (104). Knowing that the lungs are a common site for pulmonary metastasis, aerosolized GM-CSF has been tested and while it is safe (83–85, 105), it did not improve outcomes for patients with advanced sarcomas (83–85, 105). Similarly, subcutaneous GM-CSF was assessed in a phase II study for 18 pediatric patients with EWS after radiation with no significant difference in 5-year EFS between the treatment group and controls (see **Table 1**) (86).

Alternative methods of delivering intra-tumoral M1 polarizing cytokines have been developed with the goal of

minimizing global toxicities associated with exogenous cytokine administration. Innovative methods, such as adoptive transfer of macrophages harboring a soft discoidal particle ("backpack") that contains the cytokine payload, have been described. In recently published research, phagocytosis-resistant IFN-γ secreting macrophage "backpacks" is composed of external polymer layers sandwiching an IFN-γ core and a cell-adhesive layer which avidly binds to bone marrow derived macrophages. Adoptive transfer of macrophages carrying IFN-γ secreting backpacks into solid tumors maintained their M1 phenotype despite the immunosuppressive TME and also repolarized endogenous M2 TAMs toward an M1 phenotype. This was also associated with decreased tumor volume and lung metastases *in vivo* (106). Further studies into the function, feasibility, and toxicity of these and similar alternative delivery methods are needed as they seem promising as a means of avoiding systemic administration of exogenous cytokines.

MACROPHAGE PHAGOCYTOSIS

Efferocytosis is a tolerogenic phagocytotic process characterized by clearance of auto-antigen (or "self") present on apoptotic cells and suppressing T cell activation. Physiologically, efferocytosis is thought to be critical in the maintenance of self-tolerance and the prevention of autoimmunity. However, in the TME, the otherwise normal TAM process of efferocytosis diminishes immunity through phagocytic clearance of tumor antigen and suppression of T cell cytolytic function, thereby creating a TME supportive of immune evasion and subsequent tumor survival and metastasis. This may be especially relevant in settings of high cell turnover, such as malignancies which are characterized by spontaneous apoptosis due to a myriad of circumstances associated with cancer. Therefore, interfering in the multiple steps involved in efferocytosis may be a novel therapeutic approach with the potential for therapeutic benefit.

Migration Toward "Find-Me" Signals

Efferocytosis of apoptotic debris is a series of coordinated events, including chemotaxis, recognition and binding of the apoptotic particle, and ingestion. This first step of the sequence includes the secretion of chemoattractant "find-me" signals by a dying cell, including lysophosphatidylcholine (LPC) (107, 108), sphingosine-1-phosphate (S1P) (109), C-X3-C motif chemokine ligand 1 (CX3CL1) (110, 111) and nucleotides (112). Intracellular LPC and S1P are released by apoptotic cells (109, 113), while CX3CL1 is a membrane-associated protein which is cleaved by matrix metalloproteases (MMPs) during inflammation, releasing the soluble protein that acts as a chemokine (114). Nucleotides, specifically adenosine triphosphate (ATP) and uridine-5′-triphosphate (UTP) are released into the extracellular space following caspase-dependent activation. These molecules are recognized by receptors on monocytes and macrophages and result in migration to the area of cellular damage (110, 112, 115). Preclinical studies in RMS and osteosarcoma tumors support the importance of these mechanisms in pediatric sarcoma and have confirmed upregulated expression of bioactive lipids such as S1P, LPC, and lysophosphatidic acid (an LPC cleavage product) in bone marrow extracts (a common site of sarcoma metastasis) by mass spectrometry following radiation and chemotherapy (116, 117). Further clinical studies are required to evaluate the utility of these "find-me" signals as prognostic biomarkers or therapeutic targets for pediatric sarcomas.

Expression of "Eat-Me" Signals

Tumor cells may evade immune-mediated attack through downregulation of "eat-me" signals. "Eat-me" signals, such as phosphatidylserine (PS) and calreticulin (CRT), are externalized on dying cell surfaces, tagging them for removal by phagocytes. PS is a phospholipid normally localized to the inner membrane of the lipid bilayer in healthy cells; however, during apoptosis, PS accumulates on the cell surface. Similarly, CRT is also exposed on the cell surface during apoptotic stress. CRT interacts with PS and binds the complement C1q protein that serves as both bridging molecule and a PS-binding protein. CRT then binds the SRF-1 endocytic receptor found on macrophages to facilitate phagocytosis of apoptotic cells (118, 119). It is also known that macrophages can utilize their own CRT to enhance phagocytosis of tumor cells (120). Preclinical studies have shown that high expression of PS on EWS tumors increased their sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated cell death (121). Additional studies incubating alveolar and embryonal RMS cells with doxorubicin demonstrated enhanced CRT expression and increased phagocytosis of these RMS cells (4). Other studies are examining the use of "eat-me" signals, specifically CRT, as potential prognostic biomarkers in osteosarcoma (122).

"Don't Eat-Me" Receptors

To counter-balance PS or CRT expression, malignant cells may evade macrophage phagocytosis through the expression of "don't eat-me" receptors. Healthy cells express "don't eat-me" receptors CD47 and CD31 to avoid unwarranted phagocytic clearance (123, 124). CD47, the prototypical "don't eat-me" signal, is a membrane protein of the immunoglobulin (Ig) superfamily, present on most cells of the body. Ligation of CD47 with the ssignal regulatory protein alpha (SIRPα) protein on macrophages leads to phosphorylation of immunoreceptor tyrosine-based inhibition (ITIM) motifs and a significant inhibitory signaling cascade, characterized by the downstream protooncogene SRC, protein tyrosine phosphatase non-receptor type 6 (PTPN6), and protein tyrosine phosphatase non-receptor type 11 (PTPN11) phosphatases, which inhibit the buildup of myosin-IIA, and prevent the cellular structural changes needed for phagocytosis (2, 125–130). Activation of SIRPα has also been found to mediate M2 macrophage polarization, through regulation of the Notch signaling pathway (131–133). Conversely, when the SIRPα is blocked, TAMs portend a M1 phenotype (133).

Previous work in experimental models of hematologic and solid malignancies have identified CD47 and SIRPα as potential therapeutic targets, whereby blocking this axis (predominantly

using anti-CD47 mAb) demonstrated increased phagocytosis of cancer cells by macrophages (2, 4, 5, 126, 128, 134) and M1 polarization (131). Increased phagocytosis of human RMS cells was observed *in vitro* when macrophages were treated with anti-CD47 monoclonal antibody (4). In murine studies of osteosarcoma, CD47 blockade decreased tumor progression, increased macrophage infiltration into the tumor, and increased overall survival (2, 5). Currently, there are no open clinical trials targeting the CD47-SIRPα pathway for pediatric sarcomas. However, in adults, there are several open clinical trials evaluating the safety profile and efficacy of anti-CD47 monoclonal antibody (Hu5F9-G4) in a variety of solid and hematologic malignancies (NCT02953509, NCT03248479, NCT03922477, NCT03869190).

Engulfment and Efferocytosis

Simply put, phagocytosis occurs when the balance of "eat me" signals is greater than the "don't eat me" signals. Recognition of "eat-me" signals by professional phagocytes occurs through multiple receptors, such as TYRO3, AXL, and MERTK receptor tyrosine kinases and the T-cell immunoglobulin and mucin domain (TIM) receptor family (TIM-3 and TIM-4). Of these, MERTK is the prototypic efferocytosis receptor, given its involvement in the recognition, tethering and engulfment of apoptotic cells, and subsequent generation of immune tolerance through M2 polarization and T cell suppression (135–138). Following apoptotic cell ingestion, MERTK phosphorylation suppresses NF-κB nuclear translocation, leading to diminished type 1 cytokine production (*e.g.* TNF-α and IL-12) (139–141). Conversely, inhibition of MERTK in preclinical studies has shown decreased leukemia-associated macrophage expression of inhibitory checkpoint ligands, including PD-L1 and PD-L2 (discussed below), demonstrating its role in immune tolerance (142). Drugs targeting MERTK have been developed as agents for both reversing cancer progression and cancer immune evasion (143). Pre-clinical studies of MERTK inhibitors in murine solid tumor models have shown decreased tumor growth and increased CTL infiltration (144), while others demonstrated a more profound effect when MerTK inhibition is used in combination with radiation therapy (145).

TYRO3, AXL, and MERTK receptors do not bind to PS directly, rather they use the plasma circulating and locally secreted molecules, protein S (PROS1) and growth arrest specific 6 (GAS6) to provide a bridge to PS. PROS1 binds more specifically to MERTK and GAS6 binds to MERTK, TYRO3, and AXL (146–150). PROS1 and GAS6 are elevated in EWS tumor patient samples, providing increased ligand for efferocytosis to occur (151, 152). Antibodies acting as ligand sinks to bind and inactivate these bridging molecules have been evaluated in preclinical studies but are not yet clinically available (153–155).

TIM family of proteins, TIM-3 and TIM-4, act as PS receptors on macrophages to facilitate the clearance of apoptotic cells (135, 156, 157). On macrophages, TIM-4 works in conjunction with MERTK to mediate tethering and binding of apoptotic cells (156, 158) (159–161). TIM-3, a known co-inhibitory receptor on T cells, is also expressed on antigen presenting cells such as macrophages, aids in the binding and phagocytosis of apoptotic cells through the FG loop in the immunoglobulin variable region (IgV) domain (136, 162–164). Co-expression of TIM-3 with other immune checkpoints such as lymphocyte activating 3 (LAG3) and PD-1 on T cells has been observed in sarcoma patient samples (165); however, its expression on TAMs in sarcoma has not been explored. TIM-3 antibodies are being clinically tested and may be useful in both augmenting T cell activation, as well as diminishing the tolerogenic effects of efferocytosis (166, 167).

Bisphosphonates are a class of drugs designed to inhibit osteoclast activity to prevent loss of bone density in osteoporosis; however, they also suppress macrophage phagocytosis, decrease macrophage recruitment to tumor sites, and increase apoptosis of tumor cells (168, 169). Zoledronic acid is a nitrogen-containing bisphosphate with anti-tumor activity including decreased tumor volume and bone growth in primary EWS tumors, decreased recruitment of TAMs into the tumor stroma in murine sarcoma and carcinoma models (170–172), and reduction in bone metastases EWS after administration of ZA in murine *in vivo* models (171). When combined with ifosfamide, ZA exhibited synergistic effects against tumor growth and progression in a soft tissue tumor model. These promising clinical results have led to the evaluation of ZA in pediatric sarcomas (see **Table 1**). In a phase I study of high-grade metastatic osteosarcoma patients, ZA was well tolerated when administered concurrently with multi-agent chemotherapy (87). One small clinical study evaluated the anti-tumor efficacy of ZA at standard dosing for four patients with advanced stage osteosarcoma with encouraging progression-free survival (PFS) results (88). ZA in combination with standard chemotherapy for EWS patients is currently being evaluated in a multicenter phase III randomized controlled trial (Euro-EWING2012) (89).

ANTIGEN PRESENTATION

The physical interaction during antigen presentation between macrophage and T cells plays an integral role in T cell-mediated activation and tumor cell cytolysis. Antigen presentation involves three steps, which have been described as different signals. Signal 1 is the binding of peptide-loaded MHC on antigen presenting cells (APCs), such as macrophages, to antigen-specific T cell receptors (TCRs). Signal 2 is the engagement of costimulatory ligands with their cognate receptors on T cells. Conversely, binding of inhibitory ligands (on APCs) with their cognate receptors on T cells inhibits T cell activation. Signal 3 is the secretion of cytokines by APCs which modify or amplify T cell response (165).

Co-Stimulation and Co-Inhibition

Signal 1, consisting of the MHC-peptide-TCR complex, on its own is insufficient to activate T cells and may generate a tolerogenic response. However, concomitant engagement of adhesion receptors and co-stimulatory ligands (on APCs) and receptors (on T cells), known as Signal 2, creates an immunologic

connection between T cells and macrophages (165). Co-stimulatory ligands and cognate receptors are divided into two major groups: CD28/B7 receptor family and TNF/tumor necrosis factor receptor (TNFR) family.

CD28 is a T cell costimulatory receptor that transmits activating intracellular signals when it binds costimulatory ligands CD80 (B7-1) and CD86 (B7-2) on macrophages and other APCs (173, 174). In contrast to the CD28 stimulatory effects on T cells, ligation of inhibitory B7 receptors, including programmed death 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), can promote T cell suppression and/or dysfunction (175, 176). PD-1 has two known B7 ligands on macrophages, including PD-L1 and PD-L2 (177). PD-L1 is often upregulated in tumor infiltrating immune cells including macrophages (178–180). In fact, in pediatric sarcoma patient samples with greater PD-L1 expression, there was higher macrophage and DC infiltration, and a worse outcome (181–183). The treatment of murine and human macrophages with anti-PD-L1 antibodies promotes their proliferation and activation (184). Blockade of the PD-L1/PD-1 axis also enhances macrophage-mediated anti-tumor activity through efferocytosis. Although blockade of this ligand/receptor binding is typically studied for its effects on T cell function, preclinical models of PD-L1/PD-1 blockade using BALB/c *Rag2*$^{-/-}\gamma c^{-/-}$ mice (which do not have functional T cells) showed TAM-mediated efferocytosis and clearance of tumor cells (185). Disruption of the PD-1/PD-L1 axis in osteosarcoma demonstrated decreased lung metastases, reduced numbers of tumor-promoting TAMs, and increased anti-tumor M1 macrophages in the absence of T cells (183). While anti-PD-L1 or anti-PD-L2 agents have not yet been evaluated in pediatric sarcomas, in a murine model of osteosarcoma nivolumab (an anti-PD-1 monoclonal antibody) increased tumor infiltrating CD4$^+$ and CD8$^+$ T cells with greater cytotoxic potential (*i.e.*, granzyme B and IFN-γ production) and less lung metastases (186). PD-1 blockade using pembrolizumab in the SARC028 phase II study (see **Table 1**; NCT02301039) demonstrated an objective partial response (based on Response Evaluation Criteria in Solid Tumors (RECIST)) in only one osteosarcoma patient out of 22 patients and stable disease in six other patients. Of note, there were no responses in EWS patients, who typically had a low mutational burden (94). Correlative analysis of patient samples from the SARC028 study showed that pembrolizumab responders were more likely to have higher densities of activated CD8$^+$CD3$^+$PD-1$^+$ T cells and increased percentages of PD-L1$^+$ TAMs pre-treatment compared to non-responders. Pre-treatment analysis of tumors from responders also demonstrated higher densities of effector memory cytotoxic T cells and regulatory T cells compared to non-responders (187). Given the relatively mutated response of PD-1 axis blockade as monotherapy in pediatric sarcomas, combination strategies with other immune-targeted agents are currently being evaluated in clinical trials (188).

The TNFR family is the other major group of co-stimulatory molecules, which includes CD40, tumor necrosis factor receptor superfamily member 4 (TNFRSF4 or CD134), tumor necrosis factor receptor superfamily member 9 (TNFRSF9 or 4-1BB), and CD27 (165, 189, 190). The co-stimulatory receptor CD40 is a transmembrane protein expressed on monocytes, macrophages, and other antigen presenting cells (191). Its ligand, CD40 ligand (CD40L), is primarily expressed on activated T and B lymphocytes, monocytes and platelets (165). CD40 agonist monoclonal antibodies (mAbs) promote TAM M2 to M1 polarization, leading to increased production of nitric oxide and type 1 cytokines (such as IL-1, IL-12, and TNF-α), and activation of cytotoxic activity of CD8$^+$ T cells (192–195). CD40 agonism as monotherapy in advanced solid tumors had limited anti-tumor activity (196); however, use of a CD40 agonist in combination with PD-L1 and CTLA-4 blockade (see below) has shown extended survival in murine solid tumor models (197) (NCT02636725, NCT02332668).

CTLA-4 (also known as CD152) is part of the B7/CD28 family that also inhibits T cell cytotoxic function. CTLA-4 suppresses T cell activation when engaged with its respective ligands, B7-1 (CD80) and B7-2 (CD86) through inhibition of T cell receptor (TCR) signal transduction (198). Sarcoma patients have T cells with high CTLA-4 expression within the tumor and peripheral blood (199, 200). Phase I and II studies of ipilimumab, a CTLA-4 blocking mAbs, in pediatric patients with advanced solid tumors (including sarcomas) showed tolerability but no objective clinical or radiologic responses as monotherapy (NCT01445379) (95, 201). Combination therapies utilizing CTLA-4 and PD-L1 mAb blockade in early phase clinical studies have shown synergistic slowing of disease progression and extended PFS in adults with metastatic or unresectable sarcomas; however, they have not been tested in children (NCT02500797) (202, 203).

Signal 3: Cytokine Mediated Effects in the TME

Efferocytosis modulates the immune system beyond the regulation of engulfment and co-stimulation in the sarcoma TME. Intracellular signal transduction in efferocytosis favors production of tumor-permissive cytokines such as TGF-β and IL-10 consistent with the otherwise physiologic role of this process in immune tolerance, wound healing, and tissue homeostasis (204–206). TGF-β drives immunosuppressive responses in both the innate and adaptive immune systems. Within the innate immune system, TGF-β secreted by macrophages further skews cells toward an M2 alternative activation status, inhibits cytotoxic and cytokine producing activity of NK cells, and decreases migration and increases apoptosis of dendritic cells (204, 207). In the adaptive immune response, TGF-β promotes CD4$^+$ T cells to differentiate into Th2 cells and inhibits CD8$^+$ T cells antitumor activity by downregulating cytolytic genes such as granzyme B and Fas ligand (FasL), thereby reducing antitumor response (208, 209).

One method of overriding the potently suppressive TAM cytokine production is administration of exogenous type 1 cytokines (210, 211). Interferons and IL-2 are such powerful type 1 stimulators of the immune system. Dinutuximab (ch14.18, a mAb against tumor-associated disialoganglioside GD2) has

demonstrated activity against neuroblastoma cells; however, administration of the mAb alone was insufficient to prevent tumor progression, thus both GM-CSF and IL-2 were added to augment efficacy. The addition of IL-2 and GM-CSF to dinutuximab greatly enhanced ADCC by M1 macrophages; however, systemic IL-2 administration was found to have significant toxicity in patients, thus this treatment regimen may still need to be further optimized (104, 212), but could conceptually be applied to other pediatric solid tumors considering the GD2 is also expressed by sarcoma (213, 214).

TNF-α is another type 1 cytokine that has been studied for its effects on augmenting activating antigen presentation within the sarcoma TME. It is produced by classically activated macrophages and lymphocytes and was thought to be a potential immunotherapeutic agent. The majority of exogenous TNF-α administration in preclinical studies was used to mimic chronic inflammation, and thus results were not as favorable as predicted. For instance, a preclinical study of osteosarcoma demonstrated that TNF-α administration promoted the de-differentiation of osteosarcoma cells toward a primitive state, which significantly contributed to tumor growth and progression. Furthermore, blocking TNF-α using a soluble receptor (etanercept) to diminish chronic inflammation inhibited osteosarcoma tumor growth (215). Systemic administration of recombinant TNF-α with chemotherapy in an early Children's Cancer Group (CCG) phase I study was limited due to systemic toxicities and an inability to dose escalate (93). It has been suggested that further administration of cytokines may need to be targeted to the sarcoma microenvironment rather than systemic administration. As above regarding polarizing macrophages, innovative methods of cytokines delivery will be necessary to allow effective administration without the significant systemic toxicities.

METABOLISM INDUCED IMMUNOSUPPRESSION

Other mechanisms of TAM-induced immunosuppression leading to T cell dysfunction in the TME include breakdown of key metabolites, such as L-arginine and L-tryptophan, which are necessary for T cell activation and proliferation. TAMs produced and secrete arginase 1 (ARG1) and indoleamine 2,3-dioxygenase 1/2 (IDO 1/2), enzymes that catalyze and breakdown L-arginine and L-tryptophan respectively. Breakdown of these metabolites diminishes effector T cell function, thereby increasing the likelihood of cancer cell immune escape (216, 217). In fact, in a study of checkpoint inhibition in adult sarcoma patients where the response rates were lower than expected, the tumor samples had high infiltration of IDO1-expressing TAMs leading to the speculation that elimination of the suppressive TAMs is also needed (NCT02406781) (218). Supplementation with L-arginine in combination with a PD-1/PD-L1 inhibitor in a murine model of both localized and metastatic osteosarcoma increased tumor infiltrating lymphocytes and prolonged survival compared to controls (219). The use of ARG1 targeted small-molecule inhibitors demonstrated reversal of TAM-mediated immunosuppression including production of inflammatory cytokines, CTL, and NK cell tumor infiltration, T cell proliferation, expression of IFN-inducible genes, and restored cytolytic T cell function against solid malignancies *in vitro* and *in vivo* (220). While there are currently no pediatric clinical trials investigating the use of targeted agents against ARG1 and IDO 1/2, there are several studies in adult patients. Additionally, there is an open-label phase 1/2 study investigating an arginase inhibitor (INCB00158) as single or combination therapy with other immune checkpoint therapy in adult patients with advanced/metastatic solid tumors (NCT02903914).

TUMOR ANGIOGENESIS AND METASTASIS

When tumors reach a certain size, an "angiogenic switch" occurs in which mechanisms are triggered to promote angiogenesis, the formation of high-density vasculature, to increase tumor nutrient supply and improve waste removal (221). TAMs can hasten blood vessel growth through the release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF). Other cytokines released by TAMs such as TGF-β, C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 8 (CXCL8), and M-CSF further promote pro-angiogenic functions of macrophages (222–225). On the contrary, M1 polarization of TAMs results in inhibition of angiogenesis through the upregulation of anti-angiogenic factors (such as CXCL8 and IFN-β) (226).

VEGF-A is a pro-angiogenic cytokine released by TAMs (227), and has been studied in pediatric sarcomas given that angiogenesis is a critical step in solid tumor progression (228). EWS xenograft models have also showed delayed tumor progression with anti-VEGF directed therapies; however, rebound tumor growth occurred after therapy was discontinued, suggesting single agent VEGF-directed therapy may have limited success in the treatment of pediatric sarcomas (229, 230).

Once angiogenesis has been established, this allows for further tumor progression and metastasis. Metastasis is a complex multi-step process, which starts with tumor cells migrating and intravasating into the vasculature, circulating in the blood stream, eventual extravasation at target organs, and subsequent invasion and growth to establish disease. This complex process requires not only circulating tumor cells, but also requires the close cooperation of perivascular cells, endothelial cells, as well a variety of immune cells including macrophages.

CSF-1 is a chemokine that stimulates macrophage motility/migration, maturation, and survival, and has been implicated in metastasis. Its contribution to metastasis formation was demonstrated in a mammary cancer model where paracrine secretion of CSF-1 by tumor cells stimulated TAMs to migrate and provide a tract for tumors cells to follow along and invade normal tissue and vasculature (223). Congruent with this,

immunohistochemistry examination of soft tissue tumor patient samples showed increased expression of CSF-1 (M-CSF) and colony stimulating factor receptor (CSF1R) in more aggressive, higher histologic grade tumors (231). Additionally, CSF-1 mediated mobilization of macrophages and other hematopoietic stem and progenitor cells (HSPCs) are thought to be integral to the formation of the pre-metastatic niche for sarcoma cells at distant sites in the body. In an embryonal RMS murine model, HSPCs were found to be elevated in the peripheral blood during formation of the pre-metastatic niche and contributed to tumor-promoting immunosuppressive myeloid subsets at metastatic sites. Similarly, peripheral blood samples from RMS patients had elevated circulating HSPCs, and patients at greatest risk of metastases had the highest levels of circulating HSPCs at the time of diagnosis (232). Because CSF-1 is essential for TAM migration and maturation, strategic targeting of its receptor (CSF1R) has been explored (233–235). Mice bearing CSF-1 negative neuroblastoma xenografts showed decreased TAM infiltration and angiogenesis, compared to mice with CSF-1 expressing xenografts. Inhibition of CSF1R in neuroblastoma decreased TAM infiltration, improved T cell function, and decreased tumor progression compared to controls (236, 237).

Furthermore, metastasis-associated macrophages (MAMs) are recruited to tumor sites through C-C motif chemokine ligand 2 (CCL2) secretion from tumor cells, a chemokine that mediates monocyte migration from bone marrow to tissue sites through interaction with the macrophage CCL2 receptor, C-C motif chemokine receptor 2 (CCR2) (238). These MAMs secrete additional CCL2 to further augment TAM recruitment to metastatic sites, and CCL3 which instigates tumor seeding at distant sites (239). *In vivo* anti-CCL2 antibody treatment reduced the number of MAMs at metastatic sites and reduced overall tumor burden in breast cancer models (240, 241). Collectively, these studies demonstrate macrophages play roles in the development of metastases and soft tissue infiltration and are potential targets in pediatric sarcomas.

In the clinical setting, attempts have been made to combine therapies targeting tumor angiogenesis and metastasis. For example, bevacizumab (anti-VEGF-A mAb) previously has been combined with conventional chemotherapy backbones, such as vincristine, irinotecan, and temozolamide (VIT), gemcitabine, docetaxel, or low dose cyclophosphamide and sorafenib have shown limited results, producing only stable disease or partial response in a subset of patients with refractory/relapsed disease (see **Table 1**) (96–100, 242, 243).

Additionally for metastasis targeted therapies, there is limited data on combining such drugs with conventional therapies like chemotherapy. Preclinical evaluations demonstrate that combination of CSF1R inhibition after radiation therapy may more effectively decrease tumor volume (244). A majority of clinical trials studying CSF1R inhibitors are in very early clinical trial phases either as monotherapy or combination therapies for the treatment of relapsed/refractory sarcomas. For example, in a phase 1 clinical trial using a CSF1R small molecule inhibitor, pexidartinib (PLX3397) in pediatric patients with refractory solid tumors (including sarcomas) and leukemias showed tolerability, and the expansion cohort is still ongoing (NCT02390752; see **Table 1**) (101). Some trials utilizing monoclonal antibodies directed at CSF-1/CSF1R in adults exhibited limited anti-tumor activity (NCT01346358) (245, 246).

Chimeric Antigen Receptor Cellular Therapies

The development and clinical use of chimeric antigen receptor (CAR) T cell therapy for the treatment of relapsed/refractory acute lymphoblastic leukemia has provided a promising new therapy option for some patients (247, 248). CAR T cell therapy for pediatric sarcomas has been centered on the development of a CAR directed against GD-2, which is overexpressed on pediatric sarcoma patient samples, with an especially high predominance on osteosarcoma primary and metastatic lesions (249). However, despite the efficacy of CAR therapy in treating hematological malignancies, use of CAR T cell therapy in sarcomas has been more challenging. This is partly due to the difficulty of T cell homing, tumor penetration, and the presence of inhibitory cell subsets in the microenvironment, including infiltrating TAMs that inhibit T cell function.

To overcome such challenges, researchers have explored methods of inhibiting infiltrating suppressor myeloid cells (including TAMs and MDSCs) alongside CAR platforms. In preclinical models, use of all-trans retinoic acid—which differentiated infiltrating myeloid cells, lessening their suppressor function—was found to significantly increase the efficacy of GD-2 directed CAR T cells in pediatric sarcoma models (249). Similar solid tumor models with high levels of TAM or MDSC infiltration found that inhibition of CSF1R increased the efficacy of adoptively transferred T cells (250). As an alternative strategy to mitigate the T cell suppressive effects of TAMs, some groups have engineered their CAR T cells to express cytokines that will lead to TAM M1 repolarization, including IL-12 and IL-18 (251, 252).

Interestingly, the idea of inhibiting TAMs has also been evaluated using a CAR T cell directed against the TAMs themselves. In preclinical models, CAR T cells directed against folate receptor β (FRβ), which is highly expressed by M2 macrophages, lead to cytolysis of M2 macrophages; however, this has not yet been assessed in pediatric sarcomas (253).

Though unrelated to targeting TAMs in pediatric sarcoma, it is notable that some groups are looking into harnessing the infiltrative properties of macrophages by engineering CAR-Macrophages (CAR-Ms). This therapy could be used to direct phagocytic anti-tumor immunity against tumor antigen expressing cells (*e.g.* human epidermal growth factor receptor (HER2), mesothelin) or use alongside CAR T cell therapies to improve T cell penetration into the sarcoma through ECM breakdown (254, 255).

Given the rising interest in cellular therapy to treat malignancies, targeting TAMs and their closely related MDSC populations in the TME will become increasingly important. Similar approaches to inhibiting TAMs in combination with immunotherapy in development include use of bi- and tri-valent

T cell engagers (BiTEs, TriTEs) to deplete CD206 and FRβ expressing TAMs, inhibition of CXCR2 alongside T cell immunotherapy (*e.g.* nivolumab), or TAM repolarization (to an M1 phenotype) using tyrosine kinase inhibitors (256)

Research on TAM-targeting CAR T cells, TAM repolarizing agents or harnessing effector function of CAR-Ms is rapidly evolving. Further work is required to study the potential use of TAM inhibition in conjunction with immunotherapy in sarcomas to further boost anti-tumor immunity.

CONCLUSIONS

It is evident there is remarkable growth in the field of oncologic immunotherapy originating from overall improved understanding of the interaction of cancer cells, TME, and the host immune system. To enhance responses against pediatric sarcomas, new immunotherapy targets and rational combinations of existing immunotherapeutic agents are being investigated. As one of the major components of the TME, TAMs play an intricate role in the regulation of immune suppression within the tumor microenvironment, augmenting angiogenesis, and promoting tumor metastasis formation. All of these are growing areas of research for potential targets in the treatment of pediatric sarcomas. In this review, we discussed the numerous roles TAMs play in driving the immunosuppressive, tumor-promoting environment in the TME, as well as in promoting metastasis, and how this may be reversed in pediatric sarcomas. TAMs are an emerging novel target that has the potential to circumvent immune evasion and hopefully improve survival for pediatric sarcoma patients.

AUTHOR CONTRIBUTIONS

JK and AL-S conducted extensive literature review on this subject. JK AL-S, MV, and MH wrote, critically revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institutes of Health (5K08CA222699-03, AL-S), The V Foundation (AL-S), Hyundai Hope on Wheels (MH, MV, and AL-S), the St. Baldrick's Scholar Award (MH) and the National Pediatric Cancer Foundation Research Grant (MH).

REFERENCES

1. Williams RF, Fernandez-Pineda I, Gosain A. Pediatric Sarcomas. *Surg Clin North Am* (2016) 96(5):1107–25. doi: 10.1016/j.suc.2016.05.012
2. Xu JF, Pan XH, Zhang SJ, Zhao C, Qiu BS, Gu HF, et al. CD47 blockade inhibits tumor progression human osteosarcoma in xenograft models. *Oncotarget* (2015) 6(27):23662–70. doi: 10.18632/oncotarget.4282
3. Edris B, Weiskopf K, Volkmer AK, Volkmer JP, Willingham SB, Contreras-Trujillo H, et al. Antibody therapy targeting the CD47 protein is effective in a model of aggressive metastatic leiomyosarcoma. *Proc Natl Acad Sci U S A* (2012) 109(17):6656–61. doi: 10.1073/pnas.1121629109
4. Herrmann D, Seitz G, Fuchs J, Armeanu-Ebinger S. Susceptibility of rhabdomyosarcoma cells to macrophage-mediated cytotoxicity. *Oncoimmunology* (2012) 1(3):279–86. doi: 10.4161/onci.18612
5. Mohanty S, Aghighi M, Yerneni K, Theruvath JL, Daldrup-Link HE. Improving the efficacy of osteosarcoma therapy: combining drugs that turn cancer cell 'don't eat me' signals off and 'eat me' signals on. *Mol Oncol* (2019) 13(10):2049–61. doi: 10.1002/1878-0261.12556
6. Ferrari A, Sultan I, Huang TT, Rodriguez-Galindo C, Shehadeh A, Meazza C, et al. Soft tissue sarcoma across the age spectrum: a population-based study from the Surveillance Epidemiology and End Results database. *Pediatr Blood Cancer* (2011) 57(6):943–9. doi: 10.1002/pbc.23252
7. Crist WM, Anderson JR, Meza JL, Fryer C, Raney RB, Ruymann FB, et al. Intergroup rhabdomyosarcoma study-IV: results for patients with nonmetastatic disease. *J Clin Oncol* (2001) 19(12):3091–102. doi: 10.1200/JCO.2001.19.12.3091
8. Arndt CA, Stoner JA, Hawkins DS, Rodeberg DA, Hayes-Jordan AA, Paidas CN, et al. Vincristine, actinomycin, and cyclophosphamide compared with vincristine, actinomycin, and cyclophosphamide alternating with vincristine, topotecan, and cyclophosphamide for intermediate-risk rhabdomyosarcoma: children's oncology group study D9803. *J Clin Oncol* (2009) 27(31):5182–8. doi: 10.1200/JCO.2009.22.3768
9. Stevens MC, Rey A, Bouvet N, Ellershaw C, Flamant F, Habrand JL, et al. Treatment of nonmetastatic rhabdomyosarcoma in childhood and adolescence: third study of the International Society of Paediatric Oncology–SIOP Malignant Mesenchymal Tumor 89. *J Clin Oncol* (2005) 23(12):2618–28. doi: 10.1200/JCO.2005.08.130
10. Oberlin O, Rey A, Lyden E, Bisogno G, Stevens MC, Meyer WH, et al. Prognostic factors in metastatic rhabdomyosarcomas: results of a pooled analysis from United States and European cooperative groups. *J Clin Oncol* (2008) 26(14):2384–9. doi: 10.1200/JCO.2007.14.7207
11. NA Howlader N, M Krapcho, D Miller, A Brest, M Yu, J Ruhl, et al eds. *SEER Cancer Statistics Review, 1975-2016*. National Cancer Institute DCCPS, Surveillance Research Program (2018). Available at: https://seer.cancer.gov/archive/csr/1975_2016/#citation. updated posted to the SEER web site, April 2019.
12. Paulino AC. Late effects of radiotherapy for pediatric extremity sarcomas. *Int J Radiat Oncol Biol Phys* (2004) 60(1):265–74. doi: 10.1016/j.ijrobp.2004.02.001
13. Brown TR, Vijarnsorn C, Potts J, Milner R, Sandor GG, Fryer C. Anthracycline induced cardiac toxicity in pediatric Ewing sarcoma: a longitudinal study. *Pediatr Blood Cancer* (2013) 60(5):842–8. doi: 10.1002/pbc.24404
14. Heyn R, Raney RBJr., Hays DM, Tefft M, Gehan E, Webber B, et al. Late effects of therapy in patients with paratesticular rhabdomyosarcoma. Intergroup Rhabdomyosarcoma Study Committee. *J Clin Oncol* (1992) 10 (4):614–23. doi: 10.1200/JCO.1992.10.4.614
15. Rich DC, Corpron CA, Smith MB, Black CT, Lally KP, Andrassy RJ. Second malignant neoplasms in children after treatment of soft tissue sarcoma. *J Pediatr Surg* (1997) 32(2):369–72. doi: 10.1016/S0022-3468(97)90213-X
16. Pinto A, Dickman P, Parham D. Pathobiologic markers of the ewing sarcoma family of tumors: state of the art and prediction of behaviour. *Sarcoma* (2011) 2011:856190. doi: 10.1155/2011/856190
17. Barr FG, Galili N, Holick J, Biegel JA, Rovera G, Emanuel BS. Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. *Nat Genet* (1993) 3(2):113–7. doi: 10.1038/ng0293-113
18. Clark J, Rocques PJ, Crew AJ, Gill S, Shipley J, Chan AM, et al. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. *Nat Genet* (1994) 7 (4):502–8. doi: 10.1038/ng0894-502
19. Chmielecki J, Bailey M, He J, Elvin J, Vergilio JA, Ramkissoon S, et al. Genomic Profiling of a Large Set of Diverse Pediatric Cancers Identifies

Known and Novel Mutations across Tumor Spectra. *Cancer Res* (2017) 77 (2):509–19. doi: 10.1158/0008-5472.CAN-16-1106
20. Shern JF, Chen L, Chmielecki J, Wei JS, Patidar R, Rosenberg M, et al. Comprehensive genomic analysis of rhabdomyosarcoma reveals a landscape of alterations affecting a common genetic axis in fusion-positive and fusion-negative tumors. *Cancer Discov* (2014) 4(2):216–31. doi: 10.1158/2159-8290.CD-13-0639
21. Tirode F, Surdez D, Ma X, Parker M, Le Deley MC, Bahrami A, et al. Genomic landscape of Ewing sarcoma defines an aggressive subtype with co-association of STAG2 and TP53 mutations. *Cancer Discov* (2014) 4 (11):1342–53. doi: 10.1158/2159-8290.CD-14-0622
22. Crompton BD, Stewart C, Taylor-Weiner A, Alexe G, Kurek KC, Calicchio ML, et al. The genomic landscape of pediatric Ewing sarcoma. *Cancer Discov* (2014) 4(11):1326–41. doi: 10.1158/2159-8290.CD-13-1037
23. Sayles LC, Breese MR, Koehne AL, Leung SG, Lee AG, Liu HY, et al. Genome-Informed Targeted Therapy for Osteosarcoma. *Cancer Discov* (2019) 9(1):46–63. doi: 10.1158/2159-8290.CD-17-1152
24. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. *J Allergy Clin Immunol* (2003) 112(5):973–80. doi: 10.1016/j.jaci.2003.07.003
25. Sheu TT, Chiang BL, Yen JH, Lin WC. Premature CD4+ T cell aging and its contribution to lymphopenia-induced proliferation of memory cells in autoimmune-prone non-obese diabetic mice. *PLoS One* (2014) 9(2): e89379. doi: 10.1371/journal.pone.0089379
26. Sturgeon P. Volumetric and microscopic pattern of bone marrow in normal infants and children. III. Histologic pattern. *Pediatrics* (1951) 7(6):774–81. doi: 10.1111/ijlh.12073
27. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. *Mech Ageing Dev* (2000) 117(1-3):57–68. doi: 10.1016/S0047-6374(00)00137-8
28. Rego EM, Garcia AB, Viana SR, Falcao RP. Age-related changes of lymphocyte subsets in normal bone marrow biopsies. *Cytometry* (1998) 34 (1):22–9. doi: 10.1002/(SICI)1097-0320(19980215)34:1<22::AID-CYTO4>3.0.CO;2-G
29. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* (2005) 8(3):211–26. doi: 10.1016/j.ccr.2005.08.002
30. Elpek KG, Cremasco V, Shen H, Harvey CJ, Wucherpfennig KW, Goldstein DR, et al. The tumor microenvironment shapes lineage, transcriptional, and functional diversity of infiltrating myeloid cells. *Cancer Immunol Res* (2014) 2(7):655–67. doi: 10.1158/2326-6066.CIR-13-0209
31. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* (2008) 111(8):4233–44. doi: 10.1182/blood-2007-07-099226
32. Fujiwara T, Fukushi J, Yamamoto S, Matsumoto Y, Setsu N, Oda Y, et al. Macrophage infiltration predicts a poor prognosis for human ewing sarcoma. *Am J Pathol* (2011) 179(3):1157–70. doi: 10.1016/j.ajpath.2011.05.034
33. Nabeshima A, Matsumoto Y, Fukushi J, Iura K, Matsunobu T, Endo M, et al. Tumour-associated macrophages correlate with poor prognosis in myxoid liposarcoma and promote cell motility and invasion via the HB-EGF-EGFR-PI3K/Akt pathways. *Br J Cancer* (2015) 112(3):547–55. doi: 10.1038/bjc.2014.637
34. Stahl D, Gentles AJ, Thiele R, Gutgemann I. Prognostic profiling of the immune cell microenvironment in Ewing s Sarcoma Family of Tumors. *Oncoimmunology* (2019) 8(12):e1674113. doi: 10.1080/2162402X.2019.1674113
35. Chen L, Oke T, Siegel N, Cojocaru G, Tam AJ, Blosser RL, et al. The Immunosuppressive Niche of Soft Tissue Sarcomas is Sustained by Tumor Associated Macrophages and Characterized by Intratumoral Tertiary Lymphoid Structures. *Clin Cancer Res* (2020) 26:4018–30. doi: 10.1158/1078-0432.CCR-19-3416
36. Raj SK, Kooshki M, Winters M, Russell GB, Miller LD, Laurini JA, et al. Prognostic implications of tumor associated macrophages (TAMs) in soft tissue sarcoma. *J Clin Oncol* (2019) 37(15_suppl):e22548–e. doi: 10.1200/JCO.2019.37.15_suppl.e22548
37. Stroncek DF, Ren J, Lee DW, Tran M, Frodigh SE, Sabatino M, et al. Myeloid cells in peripheral blood mononuclear cell concentrates inhibit the expansion of chimeric antigen receptor T cells. *Cytotherapy* (2016) 18 (7):893–901. doi: 10.1016/j.jcyt.2016.04.003
38. Hume DA. The mononuclear phagocyte system. *Curr Opin Immunol* (2006) 18(1):49–53. doi: 10.1016/j.coi.2005.11.008
39. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int J Mol Sci* (2017) 19 (1):92. doi: 10.3390/ijms19010092
40. Hohl TM, Rivera A, Lipuma L, Gallegos A, Shi C, Mack M, et al. Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe* (2009) 6(5):470–81. doi: 10.1016/j.chom.2009.10.007
41. Kim TS, Braciale TJ. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS One* (2009) 4(1):e4204. doi: 10.1371/journal.pone.0004204
42. Kim SY, Nair MG. Macrophages in wound healing: activation and plasticity. *Immunol Cell Biol* (2019) 97(3):258–67. doi: 10.1111/imcb.12236
43. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* (2012) 109(46):E3186–95. doi: 10.1073/pnas.1119964109
44. Deng B, Wehling-Henricks M, Villalta SA, Wang Y, Tidball JG. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *J Immunol* (2012) 189(7):3669–80. doi: 10.4049/jimmunol.1103180
45. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A. Tumor associated macrophages and neutrophils in tumor progression. *J Cell Physiol* (2013) 228(7):1404–12. doi: 10.1002/jcp.24260
46. Lee CH, Espinosa I, Vrijaldenhoven S, Subramanian S, Montgomery KD, Zhu S, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. *Clin Cancer Res* (2008) 14(5):1423–30. doi: 10.1158/1078-0432.CCR-07-1712
47. Leimgruber A, Berger C, Cortez-Retamozo V, Etzrodt M, Newton AP, Waterman P, et al. Behavior of endogenous tumor-associated macrophages assessed in vivo using a functionalized nanoparticle. *Neoplasia* (2009) 11(5):459–68, 2 p following 68. doi: 10.1593/neo.09356
48. Aghighi M, Theruvath AJ, Pareek A, Pisani LL, Alford R, Muehe AM, et al. Magnetic Resonance Imaging of Tumor-Associated Macrophages: Clinical Translation. *Clin Cancer Res* (2018) 24(17):4110–8. doi: 10.1158/1078-0432.CCR-18-0673
49. Rahman N, Pervin M, Kuramochi M, Karim MR, Izawa T, Kuwamura M, et al. M1/M2-macrophage Polarization-based Hepatotoxicity in d-galactosamine-induced Acute Liver Injury in Rats. *Toxicol Pathol* (2018) 46(7):764–76. doi: 10.1177/0192623318801574
50. Dancsok AR, Gao D, Lee AF, Steigen SE, Blay JY, Thomas DM, et al. Tumor-associated macrophages and macrophage-related immune checkpoint expression in sarcomas. *Oncoimmunology* (2020) 9(1):1747340. doi: 10.1080/2162402X.2020.1747340
51. Kather JN, Horner C, Weis CA, Aung T, Vokuhl C, Weiss C, et al. CD163+ immune cell infiltrates and presence of CD54+ microvessels are prognostic markers for patients with embryonal rhabdomyosarcoma. *Sci Rep* (2019) 9 (1):9211. doi: 10.1038/s41598-019-45551-y
52. Choo MK, Sano Y, Kim C, Yasuda K, Li XD, Lin X, et al. TLR sensing of bacterial spore-associated RNA triggers host immune responses with detrimental effects. *J Exp Med* (2017) 214(5):1297–311. doi: 10.1084/jem.20161141
53. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* (1995) 374(6522):546–9. doi: 10.1038/374546a0
54. Chan MP, Onji M, Fukui R, Kawane K, Shibata T, Saitoh S, et al. DNase II-dependent DNA digestion is required for DNA sensing by TLR9. *Nat Commun* (2015) 6:5853. doi: 10.1038/ncomms6853
55. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* (2003) 3 (1):23–35. doi: 10.1038/nri978

56. Kuper C, Beck FX, Neuhofer W. Toll-like receptor 4 activates NF-kappaB and MAP kinase pathways to regulate expression of proinflammatory COX-2 in renal medullary collecting duct cells. *Am J Physiol Renal Physiol* (2012) 302(1):F38–46. doi: 10.1152/ajprenal.00590.2010
57. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, et al. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol* (1999) 162(6):3256–62. doi: 10.1016/j.coi.2010.02.013
58. Keppler SJ, Rosenits K, Koegl T, Vucikuja S, Aichele P. Signal 3 cytokines as modulators of primary immune responses during infections: the interplay of type I IFN and IL-12 in CD8 T cell responses. *PLoS One* (2012) 7(7):e40865. doi: 10.1371/journal.pone.0040865
59. Liu CP, Zhang X, Tan QL, Xu WX, Zhou CY, Luo M, et al. NF-kappaB pathways are involved in M1 polarization of RAW 264.7 macrophage by polyporus polysaccharide in the tumor microenvironment. *PLoS One* (2017) 12(11):e0188317. doi: 10.1371/journal.pone.0188317
60. Tugal D, Liao X, Jain MK. Transcriptional control of macrophage polarization. *Arterioscler Thromb Vasc Biol* (2013) 33(6):1135–44. doi: 10.1161/ATVBAHA.113.301453
61. de Gaetano M, Crean D, Barry M, Belton O. M1- and M2-Type Macrophage Responses Are Predictive of Adverse Outcomes in Human Atherosclerosis. *Front Immunol* (2016) 7:275. doi: 10.3389/fimmu.2016.00275
62. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A* (2004) 101(13):4560–5. doi: 10.1073/pnas.0400983101
63. Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *J Leukoc Biol* (2006) 79(2):285–93. doi: 10.1189/jlb.0105015
64. Ragnhammar P, Frodin JE, Trotta PP, Mellstedt H. Cytotoxicity of white blood cells activated by granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor and macrophage-colony-stimulating factor against tumor cells in the presence of various monoclonal antibodies. *Cancer Immunol Immunother* (1994) 39(4):254–62. doi: 10.1007/BF01525989
65. Charak BS, Agah R, Mazumder A. Granulocyte-macrophage colony-stimulating factor-induced antibody-dependent cellular cytotoxicity in bone marrow macrophages: application in bone marrow transplantation. *Blood* (1993) 81(12):3474–9. doi: 10.1182/blood.V81.12.3474.bloodjournal81123474
66. Young DA, Lowe LD, Clark SC. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity. *J Immunol* (1990) 145(2):607–15.
67. Yao Y, Xu XH, Jin L. Macrophage Polarization in Physiological and Pathological Pregnancy. *Front Immunol* (2019) 10:792. doi: 10.3389/fimmu.2019.00792
68. Foss DL, Zilliox MJ, Murtaugh MP. Differential regulation of macrophage interleukin-1 (IL-1), IL-12, and CD80-CD86 by two bacterial toxins. *Infect Immun* (1999) 67(10):5275–81. doi: 10.1128/IAI.67.10.5275-5281.1999
69. Moganti K, Li F, Schmuttermaier C, Riemann S, Kluter H, Gratchev A, et al. Hyperglycemia induces mixed M1/M2 cytokine profile in primary human monocyte-derived macrophages. *Immunobiology* (2017) 222(10):952–9. doi: 10.1016/j.imbio.2016.07.006
70. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* (1992) 176(1):287–92. doi: 10.1084/jem.176.1.287
71. Van Dyken SJ, Locksley RM. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. *Annu Rev Immunol* (2013) 31:317–43. doi: 10.1146/annurev-immunol-032712-095906
72. Trombetta AC, Soldano S, Contini P, Tomatis V, Ruaro B, Paolino S, et al. A circulating cell population showing both M1 and M2 monocyte/macrophage surface markers characterizes systemic sclerosis patients with lung involvement. *Respir Res* (2018) 19(1):186. doi: 10.1186/s12931-018-0891-z
73. Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, et al. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science* (2002) 295(5561):1898–901. doi: 10.1126/science.1069540
74. Halpern MS, Deery DT, Flores LJ, Fujita DJ, Mason WS. Differential reactivity of serum antibody from tumor-bearing 15I5 X 7(2) chickens for cross-reactive species of endogenous retroviral envelope glycoprotein. *Virology* (1983) 124(2):318–29. doi: 10.1016/0042-6822(83)90348-3
75. Nguyen TT, Schwartz EJ, West RB, Warnke RA, Arber DA, Natkunam Y. Expression of CD163 (hemoglobin scavenger receptor) in normal tissues, lymphomas, carcinomas, and sarcomas is largely restricted to the monocyte/macrophage lineage. *Am J Surg Pathol* (2005) 29(5):617–24. doi: 10.1097/01.pas.0000157940.80538.ec
76. Buechler C, Ritter M, Orso E, Langmann T, Klucken J, Schmitz G. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* (2000) 67(1):97–103. doi: 10.1002/jlb.67.1.97
77. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* (2002) 23(11):549–55. doi: 10.1016/S1471-4906(02)02302-5
78. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* (2002) 169(10):5538–45. doi: 10.4049/jimmunol.169.10.5538
79. Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci U S A* (2003) 100(9):5336–41. doi: 10.1073/pnas.0931259100
80. Kleinerman ES, Erickson KL, Schroit AJ, Fogler WE, Fidler IJ. Activation of tumoricidal properties in human blood monocytes by liposomes containing lipophilic muramyl tripeptide. *Cancer Res* (1983) 43(5):2010–4.
81. Asano T, McWatters A, An T, Matsushima K, Kleinerman ES. Liposomal muramyl tripeptide up-regulates interleukin-1 alpha, interleukin-1 beta, tumor necrosis factor-alpha, interleukin-6 and interleukin-8 gene expression in human monocytes. *J Pharmacol Exp Ther* (1994) 268 (2):1032–9. doi: 10.1007/BF01517165
82. Biteau K, Guiho R, Chatelais M, Taurelle J, Chesneau J, Corradini N, et al. L-MTP-PE and zoledronic acid combination in osteosarcoma: preclinical evidence of positive therapeutic combination for clinical transfer. *Am J Cancer Res* (2016) 6(3):677–89.
83. Anderson PM, Markovic SN, Sloan JA, Clawson ML, Wylam M, Arndt CA, et al. Aerosol granulocyte macrophage-colony stimulating factor: a low toxicity, lung-specific biological therapy in patients with lung metastases. *Clin Cancer Res* (1999) 5(9):2316–23.
84. Arndt CA, Koshkina NV, Inwards CY, Hawkins DS, Krailo MD, Villaluna D, et al. Inhaled granulocyte-macrophage colony stimulating factor for first pulmonary recurrence of osteosarcoma: effects on disease-free survival and immunomodulation. a report from the Children's Oncology Group. *Clin Cancer Res* (2010) 16(15):4024–30. doi: 10.1158/1078-0432.CCR-10-0662
85. Lagmay JP, Krailo MD, Dang H, Kim A, Hawkins DS, Beaty O,3, et al. Outcome of Patients With Recurrent Osteosarcoma Enrolled in Seven Phase II Trials Through Children's Cancer Group, Pediatric Oncology Group, and Children's Oncology Group: Learning From the Past to Move Forward. *J Clin Oncol* (2016) 34(25):3031–8. doi: 10.1200/JCO.2015.65.5381
86. Luksch R, Massimino M, Cefalo G, Lombardi F, Ferrari A, Casanova M, et al. Effects of recombinant human granulocyte-macrophage colony-stimulating factor in an intensive treatment program for children with Ewing's sarcoma. *Haematologica* (2001) 86(7):753–60. doi: 10.3324/%25x
87. Goldsby RE, Fan TM, Villaluna D, Wagner LM, Isakoff MS, Meyer J, et al. Feasibility and dose discovery analysis of zoledronic acid with concurrent chemotherapy in the treatment of newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. *Eur J Cancer* (2013) 49 (10):2384–91. doi: 10.1016/j.ejca.2013.03.018
88. Conry RM, Rodriguez MG, Pressey JG. Zoledronic acid in metastatic osteosarcoma: encouraging progression free survival in four consecutive patients. *Clin Sarcoma Res* (2016) 6:6. doi: 10.1186/s13569-016-0046-2
89. Anderton J, Moroz V, Marec-Berard P, Gaspar N, Laurence V, Martin-Broto J, et al. International randomised controlled trial for the treatment of newly diagnosed EWING sarcoma family of tumours - EURO EWING 2012 Protocol. *Trials* (2020) 21(1):96. doi: 10.1186/s13063-019-4026-8

90. Creaven PJ, Cowens JW, Brenner DE, Dadey BM, Han T, Huben R, et al. Initial clinical trial of the macrophage activator muramyl tripeptide-phosphatidylethanolamine encapsulated in liposomes in patients with advanced cancer. *J Biol Response Mod* (1990) 9(5):492–8.
91. Kleinerman ES, Meyers PA, Raymond AK, Gano JB, Jia SF, Jaffe N. Combination therapy with ifosfamide and liposome-encapsulated muramyl tripeptide: tolerability, toxicity, and immune stimulation. *J Immunother Emphasis Tumor Immunol* (1995) 17(3):181–93. doi: 10.1097/00002371-199504000-00007
92. Chou AJ, Kleinerman ES, Krailo MD, Chen Z, Betcher DL, Healey JH, et al. Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. *Cancer* (2009) 115(22):5339–48. doi: 10.1002/cncr.24566
93. Seibel NL, Dinndorf PA, Bauer M, Sondel PM, Hammond GD, Reaman GH. Phase I study of tumor necrosis factor-alpha and actinomycin D in pediatric patients with cancer: a Children's Cancer Group study. *J Immunother Emphasis Tumor Immunol* (1994) 16(2):125–31. doi: 10.1097/00002371-199408000-00006
94. Tawbi HA, Burgess M, Bolejack V, Van Tine BA, Schuetze SM, Hu J, et al. Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. *Lancet Oncol* (2017) 18(11):1493–501. doi: 10.1016/S1470-2045(17)30624-1
95. Merchant MS, Wright M, Baird K, Wexler LH, Rodriguez-Galindo C, Bernstein D, et al. Phase I Clinical Trial of Ipilimumab in Pediatric Patients with Advanced Solid Tumors. *Clin Cancer Res* (2016) 22(6):1364–70. doi: 10.1158/1078-0432.CCR-15-0491
96. Slotkin EK, Magnan HD, Meyers PA, Chou AJ, Ambati SR, Wexler LH. Off-label use of bevacizumab in relapsed and refractory pediatric sarcoma patients: The Memorial Sloan Kettering Cancer Center Experience. *J Clin Oncol* (2016) 34(15_suppl):10569–. doi: 10.1200/JCO.2016.34.15_suppl.10569
97. Glade Bender JL, Adamson PC, Reid JM, Xu L, Baruchel S, Shaked Y, et al. Phase I trial and pharmacokinetic study of bevacizumab in pediatric patients with refractory solid tumors: a Children's Oncology Group Study. *J Clin Oncol* (2008) 26(3):399–405. doi: 10.1200/JCO.2007.11.9230
98. Okada K, Yamasaki K, Tanaka C, Fujisaki H, Osugi Y, Hara J. Phase I study of bevacizumab plus irinotecan in pediatric patients with recurrent/refractory solid tumors. *Jpn J Clin Oncol* (2013) 43(11):1073–9. doi: 10.1093/jjco/hyt124
99. Venkatramani R, Malogolowkin M, Davidson TB, May W, Sposto R, Mascarenhas L. A phase I study of vincristine, irinotecan, temozolomide and bevacizumab (vitb) in pediatric patients with relapsed solid tumors. *PLoS One* (2013) 8(7):e68416. doi: 10.1371/journal.pone.0068416
100. Navid F, Baker SD, McCarville MB, Stewart CF, Billups CA, Wu J, et al. Phase I and clinical pharmacology study of bevacizumab, sorafenib, and low-dose cyclophosphamide in children and young adults with refractory/recurrent solid tumors. *Clin Cancer Res* (2013) 19(1):236–46. doi: 10.1158/1078-0432.CCR-12-1897
101. Wesolowski R, Sharma N, Reebel L, Rodal MB, Peck A, West BL, et al. Phase Ib study of the combination of pexidartinib (PLX3397), a CSF-1R inhibitor, and paclitaxel in patients with advanced solid tumors. *Ther Adv Med Oncol* (2019) 11:1758835919854238. doi: 10.1177/1758835919854238
102. Roberts SS, Chou AJ, Cheung NK. Immunotherapy of Childhood Sarcomas. *Front Oncol* (2015) 5:181. doi: 10.3389/fonc.2015.00181
103. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* (2014) 41(1):14–20. doi: 10.1016/j.immuni.2014.06.008
104. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* (2010) 363(14):1324–34. doi: 10.1056/NEJMoa0911123
105. Worth LL, Lafleur EA, Jia SF, Kleinerman ES. Fas expression inversely correlates with metastatic potential in osteosarcoma cells. *Oncol Rep* (2002) 9(4):823–7. doi: 10.3892/or.9.4.823
106. Shields CW, Evans MA, Wang LL-W, Baugh N, Iyer S, Wu D, et al. Cellular backpacks for macrophage immunotherapy. *Sci Adv* (2020) 6(18):eaaz6579. doi: 10.1126/sciadv.aaz6579
107. Lee HJ, Ko HJ, Song DK, Jung YJ. Lysophosphatidylcholine Promotes Phagosome Maturation and Regulates Inflammatory Mediator Production Through the Protein Kinase A-Phosphatidylinositol 3 Kinase-p38 Mitogen-Activated Protein Kinase Signaling Pathway During Mycobacterium tuberculosis Infection in Mouse Macrophages. *Front Immunol* (2018) 9:920. doi: 10.3389/fimmu.2018.00920
108. Kao YC, Owosho AA, Sung YS, Zhang L, Fujisawa Y, Lee JC, et al. BCOR-CCNB3 Fusion Positive Sarcomas: A Clinicopathologic and Molecular Analysis of 36 Cases With Comparison to Morphologic Spectrum and Clinical Behavior of Other Round Cell Sarcomas. *Am J Surg Pathol* (2018) 42(5):604–15. doi: 10.1097/PAS.0000000000000965
109. Gude DR, Alvarez SE, Paugh SW, Mitra P, Yu J, Griffiths R, et al. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB J* (2008) 22(8):2629–38. doi: 10.1096/fj.08-107169
110. Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* (2008) 112(13):5026–36. doi: 10.1182/blood-2008-06-162404
111. Tsai WH, Shih CH, Feng SY, Li IT, Chang SC, Lin YC, et al. CX3CL1(+) microparticles mediate the chemoattraction of alveolar macrophages toward apoptotic acute promyelocytic leukemic cells. *Cell Physiol Biochem* (2014) 33(3):594–604. doi: 10.1159/000358637
112. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* (2009) 461(7261):282–6. doi: 10.1038/nature08296
113. Peter C, Waibel M, Radu CG, Yang LV, Witte ON, Schulze-Osthoff K, et al. Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J Biol Chem* (2008) 283(9):5296–305. doi: 10.1074/jbc.M706586200
114. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature* (1997) 385(6617):640–4. doi: 10.1038/385640a0
115. Sokolowski JD, Chabanon-Hicks CN, Han CZ, Heffron DS, Mandell JW. Fractalkine is a "find-me" signal released by neurons undergoing ethanol-induced apoptosis. *Front Cell Neurosci* (2014) 8:360. doi: 10.3389/fncel.2014.00360
116. Schneider G, Sellers ZP, Abdel-Latif A, Morris AJ, Ratajczak MZ. Bioactive lipids, LPC and LPA, are novel prometastatic factors and their tissue levels increase in response to radio/chemotherapy. *Mol Cancer Res* (2014) 12(11):1560–73. doi: 10.1158/1541-7786.MCR-14-0188
117. Engel N, Adamus A, Schauer N, Kuhn J, Nebe B, Seitz G, et al. Synergistic Action of Genistein and Calcitriol in Immature Osteosarcoma MG-63 Cells by SGPL1 Up-Regulation. *PLoS One* (2017) 12(1):e0169742. doi: 10.1371/journal.pone.0169742
118. Berwin B, Delneste Y, Lovingood RV, Post SR, Pizzo SV. SREC-I, a type F scavenger receptor, is an endocytic receptor for calreticulin. *J Biol Chem* (2004) 279(49):51250–7. doi: 10.1074/jbc.M406202200
119. Paidassi H, Tacnet-Delorme P, Verneret M, Gaboriaud C, Houen G, Duus K, et al. Investigations on the C1q-calreticulin-phosphatidylserine interactions yield new insights into apoptotic cell recognition. *J Mol Biol* (2011) 408(2):277–90. doi: 10.1016/j.jmb.2011.02.029
120. Feng M, Chen JY, Weissman-Tsukamoto R, Volkmer JP, Ho PY, McKenna KM, et al. Macrophages eat cancer cells using their own calreticulin as a guide: roles of TLR and Btk. *Proc Natl Acad Sci U S A* (2015) 112(7):2145–50. doi: 10.1073/pnas.1424907112
121. Van Valen F, Fulda S, Truckenbrod B, Eckervogt V, Sonnemann J, Hillmann A, et al. Apoptotic responsiveness of the Ewing's sarcoma family of tumours to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). *Int J Cancer* (2000) 88(2):252–9. doi: 10.1002/1097-0215(20001015)88:2<252::AID-IJC17>3.0.CO;2-U
122. Zhang XH, Zhang Y, Xie WP, Sun DS, Zhang YK, Hao YK, et al. Expression and significance of calreticulin in human osteosarcoma. *Cancer Biomark* (2017) 18(4):405–11. doi: 10.3233/CBM-160266
123. Schurch CM, Forster S, Bruhl F, Yang SH, Felley-Bosco E, Hewer E. The "don't eat me" signal CD47 is a novel diagnostic biomarker and potential therapeutic target for diffuse malignant mesothelioma. *Oncoimmunology* (2017) 7(1):e1373235. doi: 10.1080/2162402X.2017.1373235

124. Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* (2002) 418(6894):200–3. doi: 10.1038/nature00811
125. Liu X, Pu Y, Cron K, Deng L, Kline J, Frazier WA, et al. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat Med* (2015) 21(10):1209–15. doi: 10.1038/nm.3931
126. Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* (2010) 142(5):699–713. doi: 10.1016/j.cell.2010.07.044
127. Iribarren K, Buque A, Mondragon L, Xie W, Levesque S, Pol J, et al. Anticancer effects of anti-CD47 immunotherapy in vivo. *Oncoimmunology* (2019) 8(3):1550619. doi: 10.1080/2162402X.2018.1550619
128. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* (2009) 138(2):271–85. doi: 10.1016/j.cell.2009.05.046
129. Chao MP, Weissman IL, Majeti R. The CD47-SIRPalpha pathway in cancer immune evasion and potential therapeutic implications. *Curr Opin Immunol* (2012) 24(2):225–32. doi: 10.1016/j.coi.2012.01.010
130. Tsai RK, Discher DE. Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J Cell Biol* (2008) 180(5):989–1003. doi: 10.1083/jcb.200708043
131. Lin Y, Zhao JL, Zheng QJ, Jiang X, Tian J, Liang SQ, et al. Notch Signaling Modulates Macrophage Polarization and Phagocytosis Through Direct Suppression of Signal Regulatory Protein alpha Expression. *Front Immunol* (2018) 9:1744. doi: 10.3389/fimmu.2018.01744
132. Huang F, Zhao JL, Wang L, Gao CC, Liang SQ, An DJ, et al. miR-148a-3p Mediates Notch Signaling to Promote the Differentiation and M1 Activation of Macrophages. *Front Immunol* (2017) 8:1327. doi: 10.3389/fimmu.2017.01327
133. Wang YC, He F, Feng F, Liu XW, Dong GY, Qin HY, et al. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. *Cancer Res* (2010) 70(12):4840–9. doi: 10.1158/0008-5472.CAN-10-0269
134. Advani R, Flinn I, Popplewell L, Forero A, Bartlett NL, Ghosh N, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med* (2018) 379(18):1711–21. doi: 10.1056/NEJMoa1807315
135. Santiago C, Ballesteros A, Martinez-Munoz L, Mellado M, Kaplan GG, Freeman GJ, et al. Structures of T cell immunoglobulin mucin protein 4 show a metal-Ion-dependent ligand binding site where phosphatidylserine binds. *Immunity* (2007) 27(6):941–51. doi: 10.1016/j.immuni.2007.11.008
136. DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, et al. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *J Immunol* (2010) 184(4):1918–30. doi: 10.4049/jimmunol.0903059
137. Dransfield I, Zagorska A, Lew ED, Michail K, Lemke G. Mer receptor tyrosine kinase mediates both tethering and phagocytosis of apoptotic cells. *Cell Death Dis* (2015) 6:e1646. doi: 10.1038/cddis.2015.18
138. Shibata T, Habiel DM, Coelho AL, Kunkel SL, Lukacs NW, Hogaboam CM. Axl receptor blockade ameliorates pulmonary pathology resulting from primary viral infection and viral exacerbation of asthma. *J Immunol* (2014) 192(8):3569–81. doi: 10.4049/jimmunol.1302766
139. Sen P, Wallet MA, Yi Z, Huang Y, Henderson M, Mathews CE, et al. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* (2007) 109(2):653–60. doi: 10.1182/blood-2006-04-017368
140. Camenisch TD, Koller BH, Earp HS, Matsushima GK. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. *J Immunol* (1999) 162(6):3498–503.
141. Wallet MA, Sen P, Flores RR, Wang Y, Yi Z, Huang Y, et al. MerTK is required for apoptotic cell-induced T cell tolerance. *J Exp Med* (2008) 205(1):219–32. doi: 10.1084/jem.20062293
142. Lee-Sherick AB, Jacobsen KM, Henry CJ, Huey MG, Parker RE, Page LS, et al. MERTK inhibition alters the PD-1 axis and promotes anti-leukemia immunity. *JCI Insight* (2018) 3(21):e97941. doi: 10.1172/jci.insight.97941
143. Myers KV, Amend SR, Pienta KJ. Targeting Tyro3, Axl and MerTK (TAM receptors): implications for macrophages in the tumor microenvironment. *Mol Cancer* (2019) 18(1):94. doi: 10.1186/s12943-019-1022-2
144. Cook RS, Jacobsen KM, Wofford AM, DeRyckere D, Stanford J, Prieto AL, et al. MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. *J Clin Invest* (2013) 123(8):3231–42. doi: 10.1172/JCI67655
145. Crittenden MR, Baird J, Friedman D, Savage T, Uhde L, Alice A, et al. Mertk on tumor macrophages is a therapeutic target to prevent tumor recurrence following radiation therapy. *Oncotarget* (2016) 7(48):78653–66. doi: 10.18632/oncotarget.11823
146. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* (1995) 80(4):661–70. doi: 10.1016/0092-8674(95)90520-0
147. Lew ED, Oh J, Burrola PG, Lax I, Zagorska A, Traves PG, et al. Differential TAM receptor-ligand-phospholipid interactions delimit differential TAM bioactivities. *Elife* (2014) 3:e03385. doi: 10.7554/eLife.03385
148. Shiozawa Y, Pedersen EA, Patel LR, Ziegler AM, Havens AM, Jung Y, et al. GAS6/AXL axis regulates prostate cancer invasion, proliferation, and survival in the bone marrow niche. *Neoplasia* (2010) 12(2):116–27. doi: 10.1593/neo.91384
149. Caraux A, Lu Q, Fernandez N, Riou S, Di Santo JP, Raulet DH, et al. Natural killer cell differentiation driven by Tyro3 receptor tyrosine kinases. *Nat Immunol* (2006) 7(7):747–54. doi: 10.1038/ni1353
150. Waizenegger JS, Ben-Batalla I, Weinhold N, Meissner T, Wroblewski M, Janning M, et al. Role of Growth arrest-specific gene 6-Mer axis in multiple myeloma. *Leukemia* (2015) 29(3):696–704. doi: 10.1038/leu.2014.236
151. Carrera Silva EA, Chan PY, Joannas L, Errasti AE, Gagliani N, Bosurgi L, et al. T cell-derived protein S engages TAM receptor signaling in dendritic cells to control the magnitude of the immune response. *Immunity* (2013) 39(1):160–70. doi: 10.1016/j.immuni.2013.06.010
152. Fleuren ED, Hillebrandt-Roeffen MH, Flucke UE, Te Loo DM, Boerman OC, van der Graaf WT, et al. The role of AXL and the in vitro activity of the receptor tyrosine kinase inhibitor BGB324 in Ewing sarcoma. *Oncotarget* (2014) 5(24):12753–68. doi: 10.18632/oncotarget.2648
153. Kimani SG, Kumar S, Bansal N, Singh K, Kholodovych V, Comollo T, et al. Small molecule inhibitors block Gas6-inducible TAM activation and tumorigenicity. *Sci Rep* (2017) 7:43908. doi: 10.1038/srep43908
154. Che Mat MF, Abdul Murad NA, Ibrahim K, Mohd Mokhtar N, Wan Ngah WZ, Harun R, et al. Silencing of PROS1 induces apoptosis and inhibits migration and invasion of glioblastoma multiforme cells. *Int J Oncol* (2016) 49(6):2359–66. doi: 10.3892/ijo.2016.3755
155. Abboud-Jarrous G, Priya S, Maimon A, Fischman S, Cohen-Elisha M, Czerninski R, et al. Protein S drives oral squamous cell carcinoma tumorigenicity through regulation of AXL. *Oncotarget* (2017) 8(8):13986–4002. doi: 10.18632/oncotarget.14753
156. Wong K, Valdez PA, Tan C, Yeh S, Hongo JA, Ouyang W. Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages. *Proc Natl Acad Sci U S A* (2010) 107(19):8712–7. doi: 10.1073/pnas.0910929107
157. Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, et al. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity* (2007) 27(6):927–40. doi: 10.1016/j.immuni.2007.11.011
158. Shakhov AN, Rybtsov S, Tumanov AV, Shulenin S, Dean M, Kuprash DV, et al. SMUCKLER/TIM4 is a distinct member of TIM family expressed by stromal cells of secondary lymphoid tissues and associated with lymphotoxin signaling. *Eur J Immunol* (2004) 34(2):494–503. doi: 10.1002/eji.200324590
159. Nishi C, Yanagihashi Y, Segawa K, Nagata S. MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation. *J Biol Chem* (2019) 294(18):7221–30. doi: 10.1074/jbc.RA118.006628
160. Nishi C, Toda S, Segawa K, Nagata S. Tim4- and MerTK-mediated engulfment of apoptotic cells by mouse resident peritoneal macrophages. *Mol Cell Biol* (2014) 34(8):1512–20. doi: 10.1128/MCB.01394-13
161. Yanagihashi Y, Segawa K, Maeda R, Nabeshima YI, Nagata S. Mouse macrophages show different requirements for phosphatidylserine receptor

Tim4 in efferocytosis. *Proc Natl Acad Sci U S A* (2017) 114(33):8800–5. doi: 10.1073/pnas.1705365114
162. Gleason MK, Lenvik TR, McCullar V, Felices M, O'Brien MS, Cooley SA, et al. Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. *Blood* (2012) 119 (13):3064–72. doi: 10.1182/blood-2011-06-360321
163. Avery L, Filderman J, Szymczak-Workman AL, Kane LP. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. *Proc Natl Acad Sci U S A* (2018) 115(10):2455–60. doi: 10.1073/pnas.1712107115
164. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, et al. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood* (2009) 113(16):3821–30. doi: 10.1182/blood-2008-10-185884
165. Dancsok AR, Setsu N, Gao D, Blay JY, Thomas D, Maki RG, et al. Expression of lymphocyte immunoregulatory biomarkers in bone and soft-tissue sarcomas. *Mod Pathol* (2019) 32(12):1772–85. doi: 10.1038/s41379-019-0312-y
166. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* (2010) 207(10):2187–94. doi: 10.1084/jem.20100643
167. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* (2011) 117(17):4501–10. doi: 10.1182/blood-2010-10-310425
168. Moreau MF, Guillet C, Massin P, Chevalier S, Gascan H, Basle MF, et al. Comparative effects of five bisphosphonates on apoptosis of macrophage cells in vitro. *Biochem Pharmacol* (2007) 73(5):718–23. doi: 10.1016/j.bcp.2006.09.031
169. Rogers TL, Holen I. Tumour macrophages as potential targets of bisphosphonates. *J Transl Med* (2011) 9:177. doi: 10.1186/1479-5876-9-177
170. Coscia M, Quaglino E, Iezzi M, Curcio C, Pantaleoni F, Riganti C, et al. Zoledronic acid repolarizes tumour-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway. *J Cell Mol Med* (2010) 14(12):2803–15. doi: 10.1111/j.1582-4934.2009.00926.x
171. Zhou Z, Guan H, Duan X, Kleinerman ES. Zoledronic acid inhibits primary bone tumor growth in Ewing sarcoma. *Cancer* (2005) 104(8):1713–20. doi: 10.1002/cncr.21383
172. Odri GA, Dumoucel S, Picarda G, Battaglia S, Lamoureux F, Corradini N, et al. Zoledronic acid as a new adjuvant therapeutic strategy for Ewing's sarcoma patients. *Cancer Res* (2010) 70(19):7610–9. doi: 10.1158/0008-5472.CAN-09-4272
173. Parker D. CD80/CD86 signaling contributes to the proinflammatory response of Staphylococcus aureus in the airway. *Cytokine* (2018) 107:130–6. doi: 10.1016/j.cyto.2018.01.016
174. Subauste CS, de Waal Malefyt R, Fuh F. Role of CD80 (B7.1) and CD86 (B7.2) in the immune response to an intracellular pathogen. *J Immunol* (1998) 160(4):1831–40.
175. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med* (2009) 206(6):1327–37. doi: 10.1084/jem.20082173
176. Ojalvo LS, King W, Cox D, Pollard JW. High-density gene expression analysis of tumor-associated macrophages from mouse mammary tumors. *Am J Pathol* (2009) 174(3):1048–64. doi: 10.2353/ajpath.2009.080676
177. Calles A, Liao X, Sholl LM, Rodig SJ, Freeman GJ, Butaney M, et al. Expression of PD-1 and Its Ligands, PD-L1 and PD-L2, in Smokers and Never Smokers with KRAS-Mutant Lung Cancer. *J Thorac Oncol* (2015) 10 (12):1726–35. doi: 10.1097/JTO.0000000000000687
178. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* (2010) 116(7):1757–66. doi: 10.1002/cncr.24899
179. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res* (2007) 13(7):2151–7. doi: 10.1158/1078-0432.CCR-06-2746
180. Liu Y, Zugazagoitia J, Ahmed FS, Henick BS, Gettinger SN, Herbst RS, et al. Immune Cell PD-L1 Colocalizes with Macrophages and Is Associated with Outcome in PD-1 Pathway Blockade Therapy. *Clin Cancer Res* (2020) 26 (4):970–7. doi: 10.1158/1078-0432.CCR-19-1040
181. Koirala P, Roth ME, Gill J, Piperdi S, Chinai JM, Geller DS, et al. Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic in osteosarcoma. *Sci Rep* (2016) 6:30093. doi: 10.1038/srep30093
182. Kim C, Kim EK, Jung H, Chon HJ, Han JW, Shin KH, et al. Prognostic implications of PD-L1 expression in patients with soft tissue sarcoma. *BMC Cancer* (2016) 16:434. doi: 10.1186/s12885-016-2451-6
183. Dhupkar P, Gordon N, Stewart J, Kleinerman ES. Anti-PD-1 therapy redirects macrophages from an M2 to an M1 phenotype inducing regression of OS lung metastases. *Cancer Med* (2018) 7(6):2654–64. doi: 10.1002/cam4.1518
184. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. *Cancer Immunol Res* (2018) 6(10):1260–73. doi: 10.1158/2326-6066.CIR-17-0537
185. Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature* (2017) 545(7655):495–9. doi: 10.1038/nature22396
186. Zheng B, Ren T, Huang Y, Sun K, Wang S, Bao X, et al. PD-1 axis expression in musculoskeletal tumors and antitumor effect of nivolumab in osteosarcoma model of humanized mouse. *J Hematol Oncol* (2018) 11 (1):16. doi: 10.1186/s13045-018-0580-x
187. Keung EZ, Burgess M, Salazar R, Parra ER, Rodrigues-Canales J, Bolejack V, et al. Correlative Analyses of the SARC028 Trial Reveal an Association Between Sarcoma-Associated Immune Infiltrate and Response to Pembrolizumab. *Clin Cancer Res* (2020) 26(6):1258–66. doi: 10.1158/1078-0432.CCR-19-1824
188. Davis KL, Fox E, Merchant MS, Reid JM, Kudgus RA, Liu X, et al. Nivolumab in children and young adults with relapsed or refractory solid tumours or lymphoma (ADVL1412): a multicentre, open-label, single-arm, phase 1-2 trial. *Lancet Oncol* (2020) 21(4):541–50. doi: 10.1016/S1470-2045(20)30023-1
189. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, et al. The interaction properties of costimulatory molecules revisited. *Immunity* (2002) 17(2):201–10. doi: 10.1016/S1074-7613(02)00362-X
190. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* (2005) 202(9):1213–23. doi: 10.1084/jem.20051135
191. Carbone E, Ruggiero G, Terrazzano G, Palomba C, Manzo C, Fontana S, et al. A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. *J Exp Med* (1997) 185(12):2053–60. doi: 10.1084/jem.185.12.2053
192. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol* (2010) 22(2):231–7. doi: 10.1016/j.coi.2010.01.009
193. Georgoudaki AM, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Ostling J, et al. Reprogramming Tumor-Associated Macrophages by Antibody Targeting Inhibits Cancer Progression and Metastasis. *Cell Rep* (2016) 15 (9):2000–11. doi: 10.1016/j.celrep.2016.04.084
194. Burger D, Molnarfi N, Gruaz L, Dayer JM. Differential induction of IL-1beta and TNF by CD40 ligand or cellular contact with stimulated T cells depends on the maturation stage of human monocytes. *J Immunol* (2004) 173 (2):1292–7. doi: 10.4049/jimmunol.173.2.1292
195. Bullens DM, Kasran A, Thielemans K, Bakkus M, Ceuppens JL. CD40L-induced IL-12 production is further enhanced by the Th2 cytokines IL-4 and IL-13. *Scand J Immunol* (2001) 53(5):455–63. doi: 10.1046/j.1365-3083.2001.00900.x
196. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol* (2007) 25(7):876–83. doi: 10.1200/JCO.2006.08.3311
197. Zippelius A, Schreiner J, Herzig P, Muller P. Induced PD-L1 expression mediates acquired resistance to agonistic anti-CD40 treatment. *Cancer Immunol Res* (2015) 3(3):236–44. doi: 10.1158/2326-6066.CIR-14-0226
198. Seidel JA, Otsuka A, Kabashima K. Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations. *Front Oncol* (2018) 8:86. doi: 10.3389/fonc.2018.00086

199. Contardi E, Palmisano GL, Tazzari PL, Martelli AM, Fala F, Fabbi M, et al. CTLA-4 is constitutively expressed on tumor cells and can trigger apoptosis upon ligand interaction. *Int J Cancer* (2005) 117(4):538–50. doi: 10.1002/ijc.21155
200. Hingorani P, Maas ML, Gustafson MP, Dickman P, Adams RH, Watanabe M, et al. Increased CTLA-4(+) T cells and an increased ratio of monocytes with loss of class II (CD14(+) HLA-DR(lo/neg)) found in aggressive pediatric sarcoma patients. *J Immunother Cancer* (2015) 3:35. doi: 10.1186/s40425-015-0082-0
201. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* (2013) 369(2):122–33. doi: 10.1056/NEJMoa1302369
202. Lussier DM, Johnson JL, Hingorani P, Blattman JN. Combination immunotherapy with alpha-CTLA-4 and alpha-PD-L1 antibody blockade prevents immune escape and leads to complete control of metastatic osteosarcoma. *J Immunother Cancer* (2015) 3:21. doi: 10.1186/s40425-015-0067-z
203. D'Angelo SP, Mahoney MR, Van Tine BA, Atkins J, Milhem MM, Jahagirdar BN, et al. Nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401): two open-label, non-comparative, randomised, phase 2 trials. *Lancet Oncol* (2018) 19(3):416–26. doi: 10.1016/S1470-2045(18)30006-8
204. DeRyckere D, Lee-Sherick AB, Huey MG, Hill AA, Tyner JW, Jacobsen KM, et al. UNC2025, a MERTK Small-Molecule Inhibitor, Is Therapeutically Effective Alone and in Combination with Methotrexate in Leukemia Models. *Clin Cancer Res* (2016) 23:1481–92. doi: 10.1158/1078-0432.CCR-16-1330
205. Arlauckas SP, Garren SB, Garris CS, Kohler RH, Oh J, Pittet MJ, et al. Arg1 expression defines immunosuppressive subsets of tumor-associated macrophages. *Theranostics* (2018) 8(21):5842–54. doi: 10.7150/thno.26888
206. Brouckaert G, Kalai M, Krysko DV, Saelens X, Vercammen D, Ndlovu MN, et al. Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Mol Biol Cell* (2004) 15(3):1089–100. doi: 10.1091/mbc.e03-09-0668
207. Ito M, Minamiya Y, Kawai H, Saito S, Saito H, Nakagawa T, et al. Tumor-derived TGFbeta-1 induces dendritic cell apoptosis in the sentinel lymph node. *J Immunol* (2006) 176(9):5637–43. doi: 10.4049/jimmunol.176.9.5637
208. Maeda H, Shiraishi A. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J Immunol* (1996) 156(1):73–8.
209. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* (2005) 8(5):369–80. doi: 10.1016/j.ccr.2005.10.012
210. Watkins SK, Egilmez NK, Suttles J, Stout RD. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. *J Immunol* (2007) 178(3):1357–62. doi: 10.4049/jimmunol.178.3.1357
211. Watkins SK, Li B, Richardson KS, Head K, Egilmez NK, Zeng Q, et al. Rapid release of cytoplasmic IL-15 from tumor-associated macrophages is an initial and critical event in IL-12-initiated tumor regression. *Eur J Immunol* (2009) 39(8):2126–35. doi: 10.1002/eji.200839010
212. Ladenstein R, Potschger U, Valteau-Couanet D, Luksch R, Castel V, Yaniv I, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. *Lancet Oncol* (2018) 19(12):1617–29. doi: 10.1016/S1470-2045(18)30578-3
213. Dobrenkov K, Ostrovnaya I, Gu J, Cheung IY, Cheung NK. Oncotargets GD2 and GD3 are highly expressed in sarcomas of children, adolescents, and young adults. *Pediatr Blood Cancer* (2016) 63(10):1780–5. doi: 10.1002/pbc.26097
214. Kailayangiri S, Altvater B, Meltzer J, Pscherer S, Luecke A, Dierkes C, et al. The ganglioside antigen G(D2) is surface-expressed in Ewing sarcoma and allows for MHC-independent immune targeting. *Br J Cancer* (2012) 106(6):1123–33. doi: 10.1038/bjc.2012.57
215. Mori T, Sato Y, Miyamoto K, Kobayashi T, Shimizu T, Kanagawa H, et al. TNFalpha promotes osteosarcoma progression by maintaining tumor cells in an undifferentiated state. *Oncogene* (2014) 33(33):4236–41. doi: 10.1038/onc.2013.545
216. Mondanelli G, Iacono A, Allegrucci M, Puccetti P, Grohmann U. Immunoregulatory Interplay Between Arginine and Tryptophan Metabolism in Health and Disease. *Front Immunol* (2019) 10:1565. doi: 10.3389/fimmu.2019.01565
217. Bronte V, Murray PJ. Understanding local macrophage phenotypes in disease: modulating macrophage function to treat cancer. *Nat Med* (2015) 21(2):117–9. doi: 10.1038/nm.3794
218. Toulmonde M, Penel N, Adam J, Chevreau C, Blay JY, Le Cesne A, et al. Use of PD-1 Targeting, Macrophage Infiltration, and IDO Pathway Activation in Sarcomas: A Phase 2 Clinical Trial. *JAMA Oncol* (2018) 4(1):93–7. doi: 10.1001/jamaoncol.2017.1617
219. He X, Lin H, Yuan L, Li B. Combination therapy with L-arginine and alpha-PD-L1 antibody boosts immune response against osteosarcoma in immunocompetent mice. *Cancer Biol Ther* (2017) 18(2):94–100. doi: 10.1080/15384047.2016.1276136
220. Steggerda SM, Bennett MK, Chen J, Emberley E, Huang T, Janes JR, et al. Inhibition of arginase by CB-1158 blocks myeloid cell-mediated immune suppression in the tumor microenvironment. *J Immunother Cancer* (2017) 5(1):101. doi: 10.1186/s40425-017-0308-4
221. Hanahan D, Christofori G, Naik P, Arbeit J. Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. *Eur J Cancer* (1996) 32A(14):2386–93. doi: 10.1016/S0959-8049(96)00401-7
222. Eubank TD, Galloway M, Montague CM, Waldman WJ. Marsh CB. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J Immunol* (2003) 171(5):2637–43. doi: 10.4049/jimmunol.171.5.2637
223. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* (2004) 64(19):7022–9. doi: 10.1158/0008-5472.CAN-04-1449
224. Jeon SH, Chae BC, Kim HA, Seo GY, Seo DW, Chun GT, et al. Mechanisms underlying TGF-beta1-induced expression of VEGF and Flk-1 in mouse macrophages and their implications for angiogenesis. *J Leukoc Biol* (2007) 81(2):557–66. doi: 10.1189/jlb.0806517
225. Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. *J Biol Chem* (2009) 284(10):6038–42. doi: 10.1074/jbc.C800207200
226. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer Cell* (2011) 19(1):31–44. doi: 10.1016/j.ccr.2010.11.009
227. Lewis JS, Landers RJ, Underwood JC, Harris AL, Lewis CE. Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *J Pathol* (2000) 192(2):150–8. doi: 10.1002/1096-9896(2000)9999:9999<::AID-PATH687>3.0.CO;2-G
228. Lee TH, Bolontrade MF, Worth LL, Guan H, Ellis LM, Kleinerman ES. Production of VEGF165 by Ewing's sarcoma cells induces vasculogenesis and the incorporation of CD34+ stem cells into the expanding tumor vasculature. *Int J Cancer* (2006) 119(4):839–46. doi: 10.1002/ijc.21916
229. Dalal S, Berry AM, Cullinane CJ, Mangham DC, Grimer R, Lewis IJ, et al. Vascular endothelial growth factor: a therapeutic target for tumors of the Ewing's sarcoma family. *Clin Cancer Res* (2005) 11(6):2364–78. doi: 10.1158/1078-0432.CCR-04-1201
230. DuBois SG, Marina N, Glade-Bender J. Angiogenesis and vascular targeting in Ewing sarcoma: a review of preclinical and clinical data. *Cancer* (2010) 116(3):749–57. doi: 10.1002/cncr.24844
231. Richardsen E, Sorbye SW, Crowe JP, Yang JL, Busund LT. Expression of M-CSF and CSF-1R is correlated with histological grade in soft tissue tumors. *Anticancer Res* (2009) 29(10):3861–6.
232. Giles AJ, Reid CM, Evans JD, Murgai M, Vicioso Y, Highfill SL, et al. Activation of Hematopoietic Stem/Progenitor Cells Promotes Immunosuppression Within the Pre-metastatic Niche. *Cancer Res* (2016) 76(6):1335–47. doi: 10.1158/0008-5472.CAN-15-0204
233. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* (2013) 19(10):1264–72. doi: 10.1038/nm.3337

234. Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8(+) T cells. *Oncoimmunology* (2013) 2(12):e26968. doi: 10.4161/onci.26968
235. Yan D, Kowal J, Akkari L, Schuhmacher AJ, Huse JT, West BL, et al. Inhibition of colony stimulating factor-1 receptor abrogates microenvironment-mediated therapeutic resistance in gliomas. *Oncogene* (2017) 36(43):6049–58. doi: 10.1038/onc.2017.261
236. Abraham D, Zins K, Sioud M, Lucas T, Schafer R, Stanley ER, et al. Stromal cell-derived CSF-1 blockade prolongs xenograft survival of CSF-1-negative neuroblastoma. *Int J Cancer* (2010) 126(6):1339–52. doi: 10.1002/ijc.24859
237. Mao Y, Eissler N, Blanc KL, Johnsen JI, Kogner P, Kiessling R. Targeting Suppressive Myeloid Cells Potentiates Checkpoint Inhibitors to Control Spontaneous Neuroblastoma. *Clin Cancer Res* (2016) 22(15):3849–59. doi: 10.1158/1078-0432.CCR-15-1912
238. Sierra-Filardi E, Nieto C, Dominguez-Soto A, Barroso R, Sanchez-Mateos P, Puig-Kroger A, et al. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J Immunol* (2014) 192(8):3858–67. doi: 10.4049/jimmunol.1302821
239. Kitamura T, Qian BZ, Soong D, Cassetta L, Noy R, Sugano G, et al. CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *J Exp Med* (2015) 212 (7):1043–59. doi: 10.1084/jem.20141836
240. Kitamura T, Pollard JW. Therapeutic potential of chemokine signal inhibition for metastatic breast cancer. *Pharmacol Res* (2015) 100:266–70. doi: 10.1016/j.phrs.2015.08.004
241. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* (2011) 475(7355):222–5. doi: 10.1038/nature10138
242. Shaked Y, Kerbel RS. Antiangiogenic strategies on defense: on the possibility of blocking rebounds by the tumor vasculature after chemotherapy. *Cancer Res* (2007) 67(15):7055–8. doi: 10.1158/0008-5472.CAN-07-0905
243. Hingorani P, Eshun F, White-Collins A, Watanabe M. Gemcitabine, docetaxel, and bevacizumab in relapsed and refractory pediatric sarcomas. *J Pediatr Hematol Oncol* (2012) 34(7):524–7. doi: 10.1097/MPH.0b013e31826b9d25
244. Xu J, Escamilla J, Mok S, David J, Priceman S, West B, et al. CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer Res* (2013) 73(9):2782–94. doi: 10.1158/0008-5472.CAN-12-3981
245. Blay JY, El Sayadi H, Thiesse P, Garret J, Ray-Coquard I. Complete response to imatinib in relapsing pigmented villonodular synovitis/tenosynovial giant cell tumor (PVNS/TGCT). *Ann Oncol* (2008) 19(4):821–2. doi: 10.1093/annonc/mdn033
246. Papadopoulos KP, Gluck L, Martin LP, Olszanski AJ, Tolcher AW, Ngarmchamnanrith G, et al. First-in-Human Study of AMG 820, a Monoclonal Anti-Colony-Stimulating Factor 1 Receptor Antibody, in Patients with Advanced Solid Tumors. *Clin Cancer Res* (2017) 23 (19):5703–10. doi: 10.1158/1078-0432.CCR-16-3261
247. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* (2015) 385(9967):517–28. doi: 10.1016/S0140-6736(14)61403-3
248. Gardner RA, Finney O, Annesley C, Brakke H, Summers C, Leger K, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood* (2017) 129 (25):3322–31. doi: 10.1182/blood-2017-02-769208
249. Long AH, Highfill SL, Cui Y, Smith JP, Walker AJ, Ramakrishna S, et al. Reduction of MDSCs with All-trans Retinoic Acid Improves CAR Therapy Efficacy for Sarcomas. *Cancer Immunol Res* (2016) 4(10):869–80. doi: 10.1158/2326-6066.CIR-15-0230
250. Zhang P, Zhao S, Wu C, Li J, Li Z, Wen C, et al. Effects of CSF1R-targeted chimeric antigen receptor-modified NK92MI & T cells on tumor-associated macrophages. *Immunotherapy* (2018) 10(11):935–49. doi: 10.2217/imt-2018-0012
251. Chmielewski M, Kopecky C, Hombach AA, Abken H. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Res* (2011) 71(17):5697–706. doi: 10.1158/0008-5472.CAN-11-0103
252. Chmielewski M, Abken H. CAR T Cells Releasing IL-18 Convert to T-Bet (high) FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell Rep* (2017) 21(11):3205–19. doi: 10.1016/j.celrep.2017.11.063
253. Lynn RC, Matsuyama T, Powell DJ. Targeting FRβ+ tumor associated macrophages with car T cells in ovarian cancer. *J ImmunoTher Cancer* (2015) 3(Suppl 2):P32. doi: 10.1186/2051-1426-3-S2-P32
254. Zhang W, Liu L, Su H, Liu Q, Shen J, Dai H, et al. Chimeric antigen receptor macrophage therapy for breast tumours mediated by targeting the tumour extracellular matrix. *Br J Cancer* (2019) 121(10):837–45. doi: 10.1038/s41416-019-0578-3
255. Klichinsky M, Ruella M, Shestova O, Lu XM, Best A, Zeeman M, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nat Biotechnol* (2020) 38:947–53. doi: 10.1038/s41587-020-0462-y
256. Scott EM, Jacobus EJ, Lyons B, Frost S, Freedman JD, Dyer A, et al. Bi- and tri-valent T cell engagers deplete tumour-associated macrophages in cancer patient samples. *J Immunother Cancer* (2019) 7(1):320. doi: 10.1186/s40425-019-0807-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 10 December 2021
doi: 10.3389/fonc.2021.780264

Osteosarcoma and Metastasis

Gaohong Sheng[1], *Yuan Gao*[2], *Yong Yang*[1]* *and Hua Wu*[1]*

[1] Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Osteosarcoma is the most common primary bone malignancy in adolescents. Its high propensity to metastasize is the leading cause for treatment failure and poor prognosis. Although the research of osteosarcoma has greatly expanded in the past decades, the knowledge and new therapy strategies targeting metastatic progression remain sparse. The prognosis of patients with metastasis is still unsatisfactory. There is resonating urgency for a thorough and deeper understanding of molecular mechanisms underlying osteosarcoma to develop innovative therapies targeting metastasis. Toward the goal of elaborating the characteristics and biological behavior of metastatic osteosarcoma, it is essential to combine the diverse investigations that are performed at molecular, cellular, and animal levels from basic research to clinical translation spanning chemical, physical sciences, and biology. This review focuses on the metastatic process, regulatory networks involving key molecules and signaling pathways, the role of microenvironment, osteoclast, angiogenesis, metabolism, immunity, and noncoding RNAs in osteosarcoma metastasis. The aim of this review is to provide an overview of current research advances, with the hope to discovery druggable targets and promising therapy strategies for osteosarcoma metastasis and thus to overcome this clinical impasse.

Keywords: osteosarcoma, metastasis, microenvironment, metabolism, noncoding RNAs

OPEN ACCESS

Edited by:
Chi-Kong Li,
The Chinese University of Hong Kong, Hong Kong SAR, China

Reviewed by:
Oscar M. Tirado,
Institut d'Investigacio Biomedica de Bellvitge (IDIBELL), Spain
Dominique Heymann,
Université de Nantes, France

***Correspondence:**
Yong Yang
tjyangyong@hust.edu.cn
Hua Wu
wuhua@hust.edu.cn

Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *20 September 2021*
Accepted: *16 November 2021*
Published: *10 December 2021*

Citation:
Sheng G, Gao Y, Yang Y and Wu H (2021) Osteosarcoma and Metastasis. Front. Oncol. 11:780264. doi: 10.3389/fonc.2021.780264

INTRODUCTION

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents and characterized by mesenchymal cells or osteogenic progenitor cells producing osteoid and immature bone (1). The estimated incidence rate of OS is 2–4/million/year worldwide, with first peak at age of 15–19 years (incidence: 8–11/million/year) and second minor peak at age of >60 years (2, 3). OS most commonly occurs in the metaphysis of long extremity bone, such as distal femur, proximal tibia, proximal femur, and proximal humerus, while it rarely arise in axial skeleton and other sites. The 5-year survival rate has reached a plateau in OS patients with localized disease ranging from 60%–70% since the introduction of systematic chemotherapy (4). However, the survival rate of around 20% is still dismal in patients with metastasis (5–7). More importantly, almost all patients are presumed to have subclinical micrometastatic lesions at diagnosis, whereas only 15%–20% of newly diagnosed OS are successfully detected with metastasis (8–10).

The OS cells show a high propensity to disseminate to develop metastasis, which appears to be the most important intrinsic factor for poor outcome of OS patients (5). OS can virtually metastasize to any sites or organs, mostly to lungs and occasionally to bone or lymph nodes (6, 11). The metastatic OS cells from the primary tumor undergo a series of critical steps to colonize and grow in

the second site and finally progress into clinically detectable lesions. The biological behavior of metastasis is quite different from primary tumor with respect to cell cycle, differentiation, karyotype, metabolism and surrounding microenvironment, which is caused by differentially expressed genes, shift of molecule profiles, and interaction with microenvironment (12, 13). In the last few decades, a lot of research has been carried out to uncover potential mechanisms underlying OS metastasis and has made encouraging progress. Under the unremitting efforts, more and more biomarkers that are involved in metastasis and prognosis have been discovered. Functional experiments in cells and animal models further validate some remarkable genes and signaling pathways as well as their regulatory patterns responsible for metastasis (14–17). Based on these basic and preclinical studies, many clinical trials have also been initiated to identify novel therapeutic strategies against metastasis. Early diagnosis of metastasis especially micrometastasis will significantly innovate therapeutic modality and doubtlessly improve the prognosis of patients.

In this review, we performed a thorough literature search on OS metastasis and mainly discussed those studies that have been validated *in vivo*. Our focus was primarily on the biological behavior and underlying mechanisms of metastasis. We illustrated the panorama of OS metastasis from various perspectives, such as microenvironment, osteoclast, angiogenesis, metabolism, and immunity. Also, the role of noncoding RNAs in OS metastasis was also discussed, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs). The aim of our study is to provide an overall insight into the cross-talk regulatory network in metastasis and to identify core nodes as potential targets for the development of novel therapies. Only breakthroughs in the diagnosis and treatment of metastatic OS can further improve the survival of patients.

PROCESS OF METASTASIS

The dissemination of cancer cells from the primary tumor to a secondary site requires a set of multiple steps, and these metastatic cells exhibit completely distinct characteristics from primary tumor. The pulmonary metastasis process can be divided into three stages, including escape of cancer cells from primary tumor, transit within circulation system, and colonization and establishment of metastatic lesions in lung. Although a large number of tumor cells may gain the potential to enter this metastatic cascade, there are only a few cells that can survive to successfully form metastasis due to the limited efficiency in each step of metastatic cascade (18, 19). Schematic diagram of OS metastasis to the lung is shown in **Figure 1**.

Stage 1: Dissemination From the Primary Tumor

At the first stage of metastasis, OS cells with an invasive phenotype migrate away from primary tumor and then invade into surrounding tissues. Such process of cancer invasion is critically dependent on the destruction and degradation of basement membrane and extracellular matrix (ECM), which is catalyzed by pericellular and extracellular proteases, mainly by the matrix metalloprotease (MMP) family. Remarkably, a series of studies have suggested that many proteins and microRNAs can promote OS metastasis *via* upregulating various MMPs, including MMP-9 (20, 21), MMP-13 (22, 23), membrane-associated MT1-MMP (also called MMP14) (24), and MMP-16 (25). Cathepsin is another proteolytic enzyme to be involved in OS metastasis (26). Furthermore, some inhibitors targeting MMPs have been explored to suppress OS aggressive behavior by blocking this stage, such as tissue inhibitor of metalloproteinases (TIMPs) (27) and Nobiletin (28). In addition, the interactions between tumor cells and microenvironment such as endothelial cells (29) and mesenchymal stem cells (MSCs) (30, 31) may promote tumorigenicity, whereas interactions with primed dendritic cells (32) and natural killer (NK) cells (33) have antitumor effects. For instance, MSCs promote OS metastasis by the interaction of CCL5 from MSCs and SDF-1 from OS cells *via* autocrine/paracrine communication (31). Tumor lysate-pulsed dendritic cells have been studied in combination with other candidate strategies, such as agonist antiglucocorticoid-induced tumor necrosis factor receptor (anti-GITR) antibody (32), anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody (33), and antitransforming growth factor-β (anti-TGF-β) antibody (34), to further improve OS treatment *via* enhancing antitumor immunity. In addition, NK cells can kill OS cells, including tumor-initiating cells, through the interaction between natural killer group 2 member D (NKG2D) receptor and its ligand (NKG2DL) (35). The significance of osteoclast-mediated bone destruction and resorption has been revealed throughout the OS development and progression (36). Nevertheless, the exact role of osteoclast in OS metastasis remains controversial and subject to further clarification. A more detailed discussion would be presented in the subsequent part of osteoclasts and metastasis in this review. **Figure 1A** depicts the processes of OS cell invasion and migration at primary site.

Stage 2: Transit Within Circulation System

Firstly, OS cells need to intravasate into microvasculature such as blood vasculature by crossing endothelial cells and basement membrane and then travel within blood flow. The circulating tumor cells arrest and eventually extravasate out from blood into the target secondary organ. Several studies have investigated the interactions between tumor cells and endothelia cells and identified a few related molecules, including runt-related transcription factor 2 (RUNX2), osteopontin (OPN), urokinase-type plasminogen activator (uPAR), and formyl peptide receptor type 1 (FPR1), all of which are further shown to facilitate metastasis *in vivo*. In order to survive in blood vessels, OS cells must acquire anoikis resistance property, which is regulated by many genes (37) such as *FASN* and *ID1*. Additionally, tumor cells also encounter with various physical hemodynamic forces (e.g., fluid shear stress) during transit (38). The time and intensity of circulating tumor cells exposure to fluids will affect their fates either survival or apoptosis (39). The development of bone adapts to mechanical load, suggesting that OS cells might be sensitive to their surround physical stimuli (40, 41). Moreover, the changes in physical stimuli could not only affect biological behaviors of OS cells but also influence their

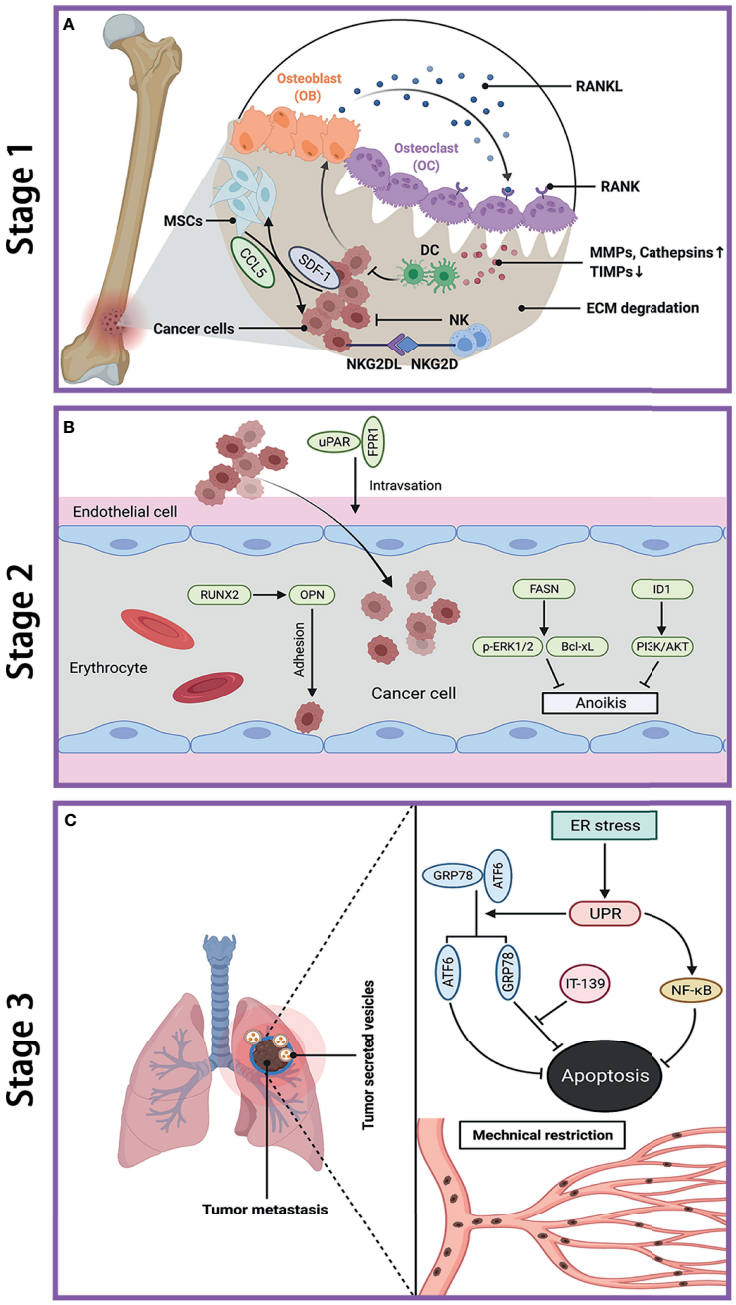


FIGURE 1 | Metastatic cascade of OS to the lung. **(A)** Stage 1: dissemination of metastatic OS cells from primary site. Cancer cells induce OB to secrete RNAKL, which binds to OC, and lead to bone resorption. The increase of MMPs and cathepsins while decrease of TIMPs cause ECM degradation. Cancer cells secret SDF-1 to recruit MSCs, which in turn promote tumor growth and metastasis by secreting CCL-5. NK cells kill cancer cell through the interaction between NKG2D receptor and NKG2D ligands (NKG2DL). **(B)** Stage 2: transfer of OS cells in blood. The interaction between uPAR and FPR1 enhances invasion of cancer cell and their entry into blood. RUNX2/OPN axis promotes adhesion of cancer cell to endothelia cell in lung. FASN and ID1 increase anoikis resistance in cancer cell by upregulating p-ERK1/2 and Bcl-xL and by activating PI3K/AKT pathway, respectively. **(C)** Stage 3: colonization of OS cells in lung. The ER stress-initiated UPR protects cancer cell from apoptosis by activating GRP78 and ATF6, as well as through NF-κB pathway. The mechanical restriction of circulating cancer cells within lung microvasculature partly accounts for the propensity of lung metastasis. Tumor-secreted vesicles reach the lung in advance and direct cancer cell to transfer to the lung. OS, osteosarcoma; OB, osteoblast; OC, osteoclast; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases; ECM, extracellular matrix; SDF-1, stromal cell-derived factor-1; MSCs, mesenchymal stem cells; CCL-5, C–C motif chemokine ligand 5; NKG2D, natural killer group 2 member D; uPAR, urokinase-type plasminogen activator; FPR1, formyl peptide receptor type 1; RUNX2, runt-related transcription factor 2; OPN, osteopontin; FASN, fatty acid synthase; ID1, inhibitor of differentiation or DNA binding; ERK, extracellular signal-regulated kinase; Bcl-xL, B-cell lymphoma-extra large; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, glucose-regulated protein 78KD; ATF6, activating transcription factor 6; IT-139, a novel small molecule that inhibit GRP78.

response to chemotherapy and radiation therapy (42, 43). The transfer of OS cells within vasculature is shown in **Figure 1B**.

Stage 3: Colonization and Establishment of Metastasis in Lung

Under a poorly defined mechanism, the majority of circulating tumor cells arrive and arrest in lung microvasculature and subsequently extravasate into lung tissues, whereas only a minority of tumor cells can survive and eventually generate detectable metastasis (19, 44). Compared with primary site, the microenvironment at the secondary site presents a lot of differences, including oxygen tension, nutrition supply, and other physiochemical features. Lung is a foreign microenvironment, where tumor cells will undergo many challenges and face various fates, including apoptosis or death (e.g., immune clearance), dormancy, and proliferation into micrometastasis. In terms of micrometastases, they also have several fates either entering into angiogenic dormancy, or regression, or proliferation to form vascularized macrometastatic lesions (45, 46).

A few studies have attempted to elucidate the potential mechanisms by which OS cells conquer the selective pressures to successfully survive and proliferate in lungs. The response of OS cells to different stresses encountered in lungs is diverse. Endoplasmic reticulum (ER) stress can alter environment in ER lumen and disturb protein folding (47). The unfolded protein response (UPR) and UPR-related signaling pathways are also identified to dysregulated in OS (48, 49). Compared with low metastatic counterparts, OS cells with high metastatic potential express a higher level of ER chaperone protein HSPA5, whose inhibition can reduce lung metastasis (50). The ER stress may even partly account for the resistance of chemotherapy in OS (51). Moreover, the mechanism remains poorly understood by which OS cells preferentially metastasize to lungs. Based on current research, the mechanical restriction of circulating tumor cells within lung microvasculature might play a crucial role in lung tropism (52, 53). Another possible explanation of lung tropism is the notion of premetastatic niche (54). Extracellular vesicles, specifically exosomes released from cancer and stromal cells provide a favorable scenario for initiating organ-directed metastasis in several cancers, including melanoma (55), gastric cancer (56), pancreatic cancer (57), and cervical squamous cell carcinoma (58). A few reviews have discussed the role of exosomes in metastatic organotropism (59, 60), among which one publication focused on bone sarcomas (61). Moreover, Hoshino et al. revealed that tumor-derived exosomes could be uptaken by organ-specific cells to prepare the premetastatic niche, which is mediated by different exosomal integrins depending on the metastatic organs (62). Although their results were mainly from breast cancer, they also profiled the proteome of OS-derived exosome. Recently, several research groups have demonstrated that OS-derived exosomes enhance OS lung metastasis and some circulating plasma exosomal biomarkers are detected to be dysregulated in metastatic OS (63–66). Another study further confirmed the preferential seeding of OS-secreted EVs in lung tissue by fluorescence microscopy with lifetime imaging *in vivo* (67). Thus, we speculate that exosomes derived from metastatic OS cells may preferentially retain in lung to create a premetastatic niche by interacting with resident cells, and thus attract OS cells to migrate to lung and support their survival. More studies are needed to investigate the biodistribution of OS-secreted exosomes and whether molecules within exosomes direct OS cells to lung. A brief description of OS lung metastasis is presented in **Figure 1C**.

MOLECULAR MECHANISMS AND PROMISING TARGETS

As mentioned above, each step of metastatic cascade is rate limited and therefore the key nodes in these steps can serve as potential targets for drug design. Some druggable targets have been summarized in other reviews based on which step the drug acts on (13, 68). Considering the clinical scenario that the majority of patients already have micrometastasis, novel therapy focusing on the later step of metastasis may be more effective. Herein, we provide an overview of biology behavior and regulatory networks of metastasis from various perspectives and summarize current research advances and explore potential targets. The data are extracted from articles, where the results have been validated in animal experiments. Based on the relevance and similarity among these data, we list the core genes and involved signaling pathways or events in **Table 1**. These genes serve as nodes to weave a biological regulatory network of OS metastasis through related pathways, which will continue to be extended and improved with further research. Promising therapy targeting such hub genes or pathways may open a new avenue to treat OS metastasis.

Microenvironment and Metastasis

Bone is a type of connective tissue with complex components, including various cells, soluble factors, and extracellular matrix (ECM). These components interact and influence with each other, by which the bone tissue maintains homeostasis in physiological conditions. The appropriate balance between bone formation and destruction determines normal structure of bone. Indeed, OS occurrence and metastatic initiation arise in such a complex bone environment. Additionally, metastatic cells also interact with surrounding microenvironment in each step of metastatic process, primarily in lung. Therefore, study on the interactions between microenvironment and metastatic cells will expand our understanding of OS biology. Increasing evidence has indicated that metastatic cells elicit and receive signals to and from microenvironment, which lead to metastasis inhibition or promotion. **Figure 2** provides a brief overview of the interactions between metastatic OS cells and microenvironment.

TP63 is a member of the well-known tumor suppressor gene *p53* family, and its splice variant *ΔNTP63* is characterized by the lack of N-terminal transactivation domain. Bid and colleagues have shown that *ΔNTP63* overexpression in OS cells increases phosphorylation of signal transducer and activator of transcription 3 (STAT3) by enhancing interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion (123). In addition, phosphorylation of STAT3 can stabilize hypoxia-inducible factor 1-alpha (HIF1-α) and induce vascular endothelial growth factor (VEGF) secretion.

TABLE 1 | Summary of genes and signaling pathways involved in osteosarcoma metastasis.

Author	Target gene	Pro/anti	Downstream pathways or events	Author	Target gene	Pro/anti	Downstream pathways or events
Ren et al. (69)	*EZR*	Pro	Lactate production, oxygen consumption↑	Han et al. (70)	*VEGF*	Pro	Meta-analysis
Khanna et al. (16)	*EZR*	Pro	MAPK↑	Jia et al. (71)	*VEGF*	Pro	Angiogenesis↑
Wan et al. (72)	*ITGB4*	Pro	Ezrin↑	Gao et al. (73)	*VEGF*	Pro	Angiogenesis↑
Ren et al. (74)	*PRKC*	Pro	Ezrin↑	Broadhead et al. (75)	*PEDF*	Anti	Angiogenesis↓
Lafleur et al. (76)	*FAS*	Anti	–	Ek et al. (77)	*PEDF*	Anti	Angiogenesis↓
Gordon et al. (78)	*FAS/FASLG*	Anti	–	Ek et al. (79)	*PEDF*	Anti	ALP, pro-α1 collagen, osteocalcin↑
Dhupkar et al. (80)	*PDCD1*	Pro	M2 macrophages, STAT-3/ERK1/2↑	Ek et al. (81)	*PEDF*	Anti	VEGF↓
Lussier et al. (82)	*PDCD1/CD274*	Pro	T-Cell immunity↓	Tang et al. (83)	*CDH4*	Pro	c-Jun/JNK↑
Gvozdenovic et al. (84)	*CD44*	Pro	Hyaluronic acid↑	Dass et al. (85)	*JUN*	Pro	Caspase-1/2/8↓
Gvozdenovic et al. (86)	*CD44*	Anti	Merlin↑	Dass et al. (87)	*JUN*	Pro	Chemosensitivity to doxorubicin↓
Xu et al. (88)	*CD47*	Pro	–	Dass et al. (89)	*JUN*	Pro	Caspase-1/2/8↓
Manara et al. (90)	*CD99*	Anti	Caveolin-1↑ c-Src↓	Sabile et al. (91)	*CCN1*	Pro	–
Adhikari et al. (92)	*CD117*	Pro	CXCR4↑	Habel et al. (93)	*CCN1*	Pro	VEGF, FGF2, PECAM, Ang, MMP-2↑ TSP-1, SPARC↓
He et al. (94)	*CD133*, *CD44*	Pro	–	Habel et al. (95)	*CCN1*	Pro	IGF1/IGF1Rβ, EMT↑
Zhang et al. (96)	*CD151*	Pro	GSK-3β/β-catenin/MMP-9↑	Tu et al. (97)	*IL6*	Pro	STAT3, PCNA↑
Kularatne et al. (98)	*CXCR4*	Pro	–	Zhang et al. (99)	*IL6*	Pro	JAK/STAT3, MAPK/ERK1/2↑
Neklyudova et al. (100)	*CXCL12*	Pro	CXCR4↑	Itoh et al. (101)	*TET2*	Pro	IL-6, MEK/ERK1/2/HIF-1α, ICAM-1↑
Dang et al. (102)	*CXCL5/CXCR2*	Pro	–	Wang et al. (103)	*IL17A/IL17RA*	Pro	VEGF, MMP-9, CXCR4, STAT3↑
Du et al. (104)	*IL8*	Pro	CXCR1/AKT↑	Ségaliny et al. (105)	*IL34*	Pro	Angiogenesis, M2 macrophages↑
Brennecke et al. (106)	*CXCR4/CXCL12*	Pro	AKT, ERK↑	Akiyama et al. (107)	*RANK-Fc*	Anti	RANK/RANKL, ERK↓ anoikis↑
Zhang et al. (108)	*VEGF*	Pro	CXCR4↑	Akiyama et al. (109)	*RANK-Fc*	Anti	RANKL, TRAP, osteoclasts↓
Surmenian et al. (110)	*CXCR7/CXCL12*	Pro	–	Lamoureux et al. (111)	*RANK-Fc*	Anti	RANK, osteolysis↓
Nigris et al. (112)	*YY1*	Pro	VEGF/CXCR4↑	Picarda et al. (113)	*TNFSF10*	Anti	Osteolysis↓ caspase-8↑
Gozo et al. (114)	*FOXC2*	Pro	CXCR4↑	Cao et al. (115)	*SP7*	Anti	Osteolysis↓
Brennecke et al. (116)	*CXCR7*	Pro	CXCL12/CXCR4	Lamora et al. (117)	*SMAD7*	Anti	TGF-β/Smad, TβRI, RANKL↓
Perissinotto et al. (118)	*CXCR4/CXCL12*	Pro	MMP-9↑	Munoz et al. (119)	*ACP5*	Anti	Osteoclasts↑
Li et al. (120)	*DNMT1*	Pro	CXCL12/CXCR4, cytotoxic T-cell homing↓	Calleja et al. (54)	*ΔNTP63α*	Pro	miR-527/665/198↓ SMAD4, TβRII (TGFBR2), KSRP (KHSRP)↑
Kimura et al. (97)	*ITGB1*	Pro	–	Gross et al. (121)	*ΔNTP63*	Pro	IL-6, CXCL8↑
Gvozdenovic et al. (122)	*ITG*	Pro	Anoikis↓ Hippo pathway↑	Bid et al. (123)	*ΔNTP63*	Pro	IL-6/8, STAT-3, HIF-1α, VEGF↑
Li et al. (124)	*NFKB*	Pro	β1 integrin↑	Li et al. (125)	*EDNRA*	Pro	MMP-2↑
Pourebrahim et al. (126)	*TP53*	Anti	Ets2, snoRNAs↓	Zhang et al. (127)	*SALL4*	Pro	Wnt/β-catenin↑
Zhang et al. (128)	*TP53*	Anti	ONZIN/CXCL5/MAPK/ERK↓	Yong et al. (129)	*LDOC1*	Anti	Wnt5a↓

(Continued)

TABLE 1 | Continued

Author	Target gene	Pro/anti	Downstream pathways or events	Author	Target gene	Pro/anti	Downstream pathways or events
Luther et al. (130)	*IGFBP5*	Anti	C-terminal domain	Wang et al. (131)	*MNAT1*	Pro	AKT1↑
Su et al. (132)	*IGFBP5*	Anti	–	Zeng et al. (133)	*ATF4*	Pro	MTA1/HDAC1↑
Wang et al. (134)	*EFEMP1*	Pro	Wnt/β-catenin, EMT↑	Lu et al. (135)	*IRX1*	Pro	CXCL14/NF-κB↑
Zhang et al. (136)	*EFEMP1*	Pro	PI3K/AKT/mTOR, EMT↑	Manara et al. (137)	*ALP*	Anti	MMP-9↓
Zhang et al. (138)	*S100A4*	Pro	MMP-9↑	Li et al. (139)	*BTG2*	Anti	PI3K/AKT↓
Fujiwara et al. (140)	*S100A4*	Pro	–	Chen et al. (141)	*SLC25A22*	Pro	AKT/FAK↑ PTEN↓
Qin et al. (142)	*TRIM2*	Pro	PI3K/PKB↑	Chien et al. (143)	*NAA10*	Pro	MMP-2↑
Chen et al. (144)	*TRIM66*	Pro	TGF-β, EMT↑	Li et al. (145)	*CDKN1B*	Pro	–
Cao et al. (146)	*WSB1*	Pro	Rac1↑ RhoGDI2↓	Munoz et al. (147)	*PLAU*	Pro	uPAR↑
Fukaya et al. (148)	*PI3K/AKT*	Pro	MMP-2/9↑	Shi et al. (149)	*CRYAB*	Pro	ERK, MMP-9↑
Liu et al. (150)	*AREG*	Pro	ICAM-1↑	Lv et al. (151)	*EZH2*	Pro	H3K27me3↑ TSSC3↓
Guo et al. (152)	*TGFBI*	Pro	α2β1 integrin, PI3K/AKT↑	Zhang et al. (128)	*USP22*	Pro	PI3K/AKT, EMT↑
Baglio et al. (153)	*TGFB*	Pro	IL-6, STAT3↑	Ren et al. (154)	*MIG7*	Pro	Vasculogenic mimicry↑
Liu et al. (155)	*BMI1*	Pro	NF-κB, MMP-9↑	Han et al. (156)	*DNMT3A*	Pro	APCDD1↓ Wnt/β-catenin, EMT↑
Naggar et al. (157)	*HACE1*	Anti	RAC1, ROS↓	Yue et al. (158)	*MAPK7*	Pro	Slug/MMP-9↑
Sun et al. (159)	*ACTL6A*	Pro	EMT↑	Contaldo et al. (160)	*IRS1*	Pro	–
Zhao et al. (161)	*SLC16A1*	Pro	NF-κB↑	Levings et al. (162)	*POU5F1*	Pro	–
Wang et al. (163)	*MTDH*	Pro	NF-κB/EFEMP1/MMP-2↑	Arlt et al. (164)	*FSCN1*	Pro	MMP-9↑
Zandueta et al. (165)	*MGP*	Pro	MMPs, TGF-β/Smad2/3↑	Long et al. (166)	*ALDOA*	Pro	MMP-2↑
Xu et al. (167)	*CEP55*	Pro	AKT, CCND1, FN1↑	Ma et al. (168)	*UBD*	Pro	HOXB9↑
Ren et al. (169)	*PHLDA1*	Pro	ERK1/2, JNK, p38↑	Niinaka et al. (170)	*AMF/GPI*	Pro	vimentin, EMT↑ E-cadherin↓
Shintani et al. (303)	*DCN*	Anti	–	Tsuru et al. (20)	*HEY1*	Pro	MMP–9↑
Yu et al. (171)	*MED19*	Pro	Cyclin D1/B1, Ki67, PCNA↑ caspase-3, PARP↓	Zhang et al. (172)	*P2RX7*	Pro	PI3K/AKT/GSK3β/β-catenin, mTOR/HIF1α/VEGF↑
Hou et al. (173)	*CCN2*	Pro	integrin/FAK/PI3K/AKT/NF-κB, VCAM-1, αvβ3↑	Zhao et al. (174)	*SPARCL1*	Anti	LRP5/6/Wnt/β-catenin, FZDRs, CXCL5, macrophages↑
McManus et al. (175)	*HES4*	Pro	–	Dai et al. (176)	*RANBP9-PHLDA2*	Anti	AKT↓ anoikis↑
Zhang et al. (177)	*NOTCH*	Pro	HES1↑	Brun et al. (178)	*FHL2*	Pro	Wnt/β-catenin↑
Hughes et al. (179)	*NOTCH*	Pro	Hes1↑	Weekes et al. (70)	*Fos/AP-1*	Pro	FGFR1, MAPK, FRS2α↑
Cheng et al. (180)	*CUL1*	Pro	MMP-9↑	Zhang et al. (181)	*SKP2*	Pro	–
Morrow et al. (182)	Metastatic variant enhancer loci	Pro	BET, AP-1, coagulation factor III/ tissue factor (F3)↑	Zhang et al. (183)	*SIRT1*	Pro	–
Zhang et al. (184)	*COPS3*	Pro	Raf-1, Beclin1, MEK/ERK, RSK, EMT, LC3-I /II↑	Lv et al. (185)	*PHLDA2*	Anti	Wnt/β-catenin/Snail/TCF-4, CD44, MMP-7, LRP5, EMT↓ GSK-3β↑
Techavichit et al. (186)	*SFRP2*	Pro	–	Zhao et al. (187)	*PHLDA2*	Anti	Src/PI3K/AKT/mTOR↓ ATG5, autophagy↑
Ma et al. (188)	*CLU*	Anti	Chemoresistance↓	Jin et al. (189)	*PRKDC*	Pro	CyclinD1, PCNA, Bcl-2↑ Bax↓

(Continued)

TABLE 1 | Continued

Author	Target gene	Pro/anti	Downstream pathways or events	Author	Target gene	Pro/anti	Downstream pathways or events
Hou et al. (190)	*TGFA/EGFR*	Pro	PI3K/AKT/NF-kB, ICAM-1↑	Tieken et al. (191)	*F3*	Pro	IL-8, CXCL-1, SNAIL, MMP-2↑
Chang et al. (192)	*SPON1*	Pro	Fak, Src, MMP-9↑	Yu et al. (193)	*MAD2*	Pro	–
Naggar et al. (194)	*YBX1*	Pro	HIF1α↑	Rubin et al. (195)	*WIF1*	Anti	Wnt↓
Hu et al. (196)	*IDH1*	Anti	–	Cantiani et al. (197)	*CAV1*	Anti	c-Src, MET↓
Sévère et al. (198)	CBL	Anti	RTK, EGFR, PDGFRα↓				

Studies using animal models of osteosarcoma metastasis are included.
Pro, the target gene promotes metastasis; anti, the target gene inhibits metastasis; "↑" upregulation; "↓" downregulation.

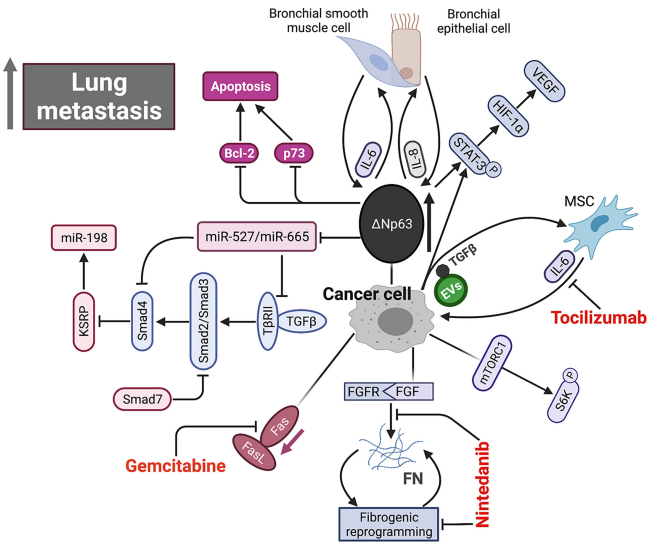


FIGURE 2 | Schematic representation of signaling pathways within microenvironment underlying OS metastasis. Aberrant overexpression of ΔNp63 in cancer cell directly drives feed-forward amplification of IL-6 and IL-8 production by the interactions between cancer cell and both primary bronchial epithelial cell and bronchial smooth muscle cell. ΔNp63 overexpression leads to elevated phosphorylation of STAT-3, which further activates HIF-1α/VEGF axis. High expression of ΔNp63 promotes cancer cell survival by inhibiting Bcl-2 and p73-depedent apoptosis. In addition, ΔNp63 represses miR-527 and miR-665, leading to the upregulation of two TGF-β effectors, Smad4 and TβRII, which inhibits anti-metastasis miR-198 by suppressing its regulatory factor, KSRP. FGF signaling initiates and FN signaling sustains fibrotic reprogramming. Nintedanib targets the pan FGFR-FN axis to inhibit OS lung metastasis. Fas-negative OS cells are selected during metastasis by evading elimination in lung where Fas ligand (FasL) is constitutively expressed. Gemcitabine appears to be a promising agent by upregulating Fas expression. EVs secreted by OS cells selectively incorporate a membrane-associated form of TGF-β and induce IL-6 production by MSCs, which in turn promotes OS progression. IL-6, interleukin-6; IL-8, interleukin-8; STAT-3, signal transducer and activator of transcription 3; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor; TGF-β, tumor growth factor β; FGF, fibroblast growth factor; FN, fibronectin; EVs, extracellular vesicle.

Combined with clinical data, these results reveal a prometastatic role of *ΔNTP63* in OS. Furthermore, the suppression of above cytokine/chemokine signaling pathways can reduce OS metastasis. In a mouse model, the inhibitions of IL-6 and C–X–C motif chemokine 8 (CXCL8, also called IL-8) significantly prolong survival by decreasing their deaths from metastasis (121). Moreover, the combination of inhibitors against IL-6 and CXCL8 achieves an intensive antimetastatic efficiency whereas each inhibitor alone only shows a modest effect. Another laboratory has reported that the metastatic OS cells expressing *ΔNTP63α* disseminate in a transforming growth factor beta (TGFβ)-rich microenvironment by upregulation of Smad4 and TβRII *via* suppressing miRNA-527/665 (120). In addition, *TGF-β* expression on the surface of extracellular vesicles (EVs), derived from OS cells, can induce the IL-6 secretion from mesenchymal stem cells (MSCs), which in turn facilitated proliferation of metastatic cells by activating STAT3. The administration of anti-IL-6 antibody disturbs this cross-talk signaling to reduce metastasis in mice (153). A research group recently has reported that myofibroblastic reprogramming of OS cells contribute to the formation of lung metastasis. This fibrotic reprogramming could be initiated by the activation of fibroblast growth factor (FGF)

signaling and sustained by the resultant fibronectin (FN) deposition. They also demonstrated the efficacy of nintedanib in disrupting lung metastasis, but not in primary bone lesion, by blocking fibrotic reprogramming through inhibiting pan FGFR-FN axis (199).

Kleinerman and colleagues have indicated a noteworthy connection between OS cells and resident cells within lung, that is, the metastatic cells expressing Fas will be eliminated by binding to ligand (FasL), which is consistently expressed in lung (76). Under such selective pressure, only those Fas-negative OS cells or cells with nonfunctional Fas signaling can evade this defense mechanism and survive in lung (200). Thus, it is feasible to find agents that can induce Fas expression on OS cells as an alternative therapeutic approach against lung metastasis. This research group has identified two agents, chemotherapeutic agent gemcitabine and histone deacetylase inhibitor entinostat, both of which induce the regression of lung metastasis in wild-type mice by upregulating Fas expression (201, 202). However, such therapeutic efficacy was not observed in FasL-deficient mice since a FasL+ lung microenvironment is a prerequisite for this treatment (78, 203, 204). Moreover, another study confirmed this result again in a canine model with lung metastasis (205). These findings suggest that incorporating lung microenvironment as part of the therapy strategy may benefit patients with established lung metastasis. As an important factor in microenvironment, EVs like tumor-derived exosomes contain various components (e.g., proteins and nucleic acids) and play an essential role in intercellular communication and metastatic progression in a variety of cancers (206). Notably, the expression profiles of EVs from OS samples with low metastatic potential are significantly distinguished from that in high metastatic potential samples by transcriptome analysis (207). A study further indicated that culture medium supplemented with exosomes would change secretome of OS cells and affect their aggressive properties (208). Interestingly, proteomic analysis of the EVs derived from highly metastatic OS cells shows the enrichment of metastasis-related proteins. The EVs from highly metastatic clonal variants of OS can even be internalized into low metastatic cells and thereby endow them with metastatic ability through horizontal phenotypic transfer (67). Also, an *in vivo* metastatic model further confirmed the potential of OS-derived EVs to promote metastasis (65). The exosomes released by OS cells, carrying programmed death-ligand 1 (PD-L1) and N-cadherin, could also stimulate pulmonary metastasis (63), and a recent study found that the plasma exosomal sentrin SUMO-specific protease 1 (SENP1) level is closely related to pulmonary metastasis in OS patients (66). As such, OS cells preferentially migrate and localize to lung directed by the EVs they have secreted, which may partly account for lung tropism of metastasis. The role of cancer-derived exosomes in cancer development and progression was systematically discussed in a review by Kok et al., mainly elaborating on the trafficking of enriched genetic signals carried by exosomal cargo in fostering cancer progression in several tumor types, including OS (209). In addition to exosomes derived from the tumor itself, exosomes from other cells such as bone marrow mesenchymal stem cells (210), adipose-derived mesenchymal stem cells (211) and tumor-associated macrophages can also affect metastasis of osteosarcoma (212, 213). OS cells and non-OS cells in tumor microenvironment could influence themselves or each other by releasing EVs through autocrine/paracrine pathways, shaping tumor microenvironment, modulating cell biological behaviors, especially the aggressiveness of OS cells. The secreted CXCLs within microenvironment selectively recruit different types of cells through binding to their transmembrane receptors. Tumor cells and leukocytes expressing CXCRs migrate following CXCL gradient (214). The CXCL/CXCR axis plays an essential role in leukocyte trafficking, immune homeostasis maintenance like T-cell homing, directional migration of tumor cells. It is well documented that the binding of CXCL12 (also known as SDF-1) to its receptor like CXCR4 or CXCR7 remarkably promotes tumor progression including OS (100, 118, 215). In primary bone site, OS epigenetically downregulates CXCL12 expression by DNMT1, impairs cytotoxic T-cell homing to the tumor site and this chemokine gradient of CXCL12 drives the metastasis of OS cells to the lung (**Figure 3A**), where CXCL12 is highly expressed. The constitutive expression of CXCL12 in lung may largely determine lung as the main site of OS metastasis (**Figure 3B**). In addition to CXCR4, CXCR7 is another receptor for CXCL12 and also participate in OS lung metastasis (110). The coexpression of CXCR7 and CXCR4 on OS cells could enhance their metastatic ability, since a chemokine gradient of CXCL12 between bone and lung is produced through CXCR7-mediated CXCL12 scavenging in primary bone site (116).Moreover, osteoprotegerin could induce SDF-1 secretion from endothelial cells, which further promotes OS development by increasing neovascularization *via* SDF-1/CXCR4 axis (216). Nigris et al. indicated that transcriptional repressor Yin Yang 1 protein (YY1) enhances metastatic potential of OS cells by activating VEGF/CXCR4 axis (112). Another study also suggested that VEGF secreted from MSCs promotes CXCR4-mediated metastasis (108). In addition, IL-8 produced by MSCs increases anoikis resistance and metastasis of OS cells by regulating CXCR1/Akt signaling pathway (104). Gozo and colleagues has demonstrated that forkhead box protein C2 (FOXC2) maintains OS cells in a stem-like state and promotes metastasis by increasing CXCR4 (114). More importantly, the inhibition of CXCR4 by its antibody (98, 106) or inhibitor (like AMD300) (217) can successfully reduce metastasis. Due to the prominent role of CXCL/CXCR axis in metastasis and its close association with angiogenesis, anoikis, and immune response, novel therapy targeting this axis might be promising for treating metastasis.

Osteoclasts and Metastasis

The progression of bone tumor leads to osteolysis (218), which in turn promotes the dissemination of tumor cells and thus forms by a vicious cycle. Breaking this vicious cycle between osteoclasts and tumor cells may be one of the promising ways to treat OS metastasis. Several studies have used RANK-Fc to perturb TNFRSF11A/TNFSF11 (also called RANK/RANKL) axis and successfully reduced metastasis by inducing anoikis and apoptosis of OS cells (107, 109, 111). In addition, other agents that can restrict bone resorption are also expected to become potential candidates to inhibit metastasis, such as inhibitors

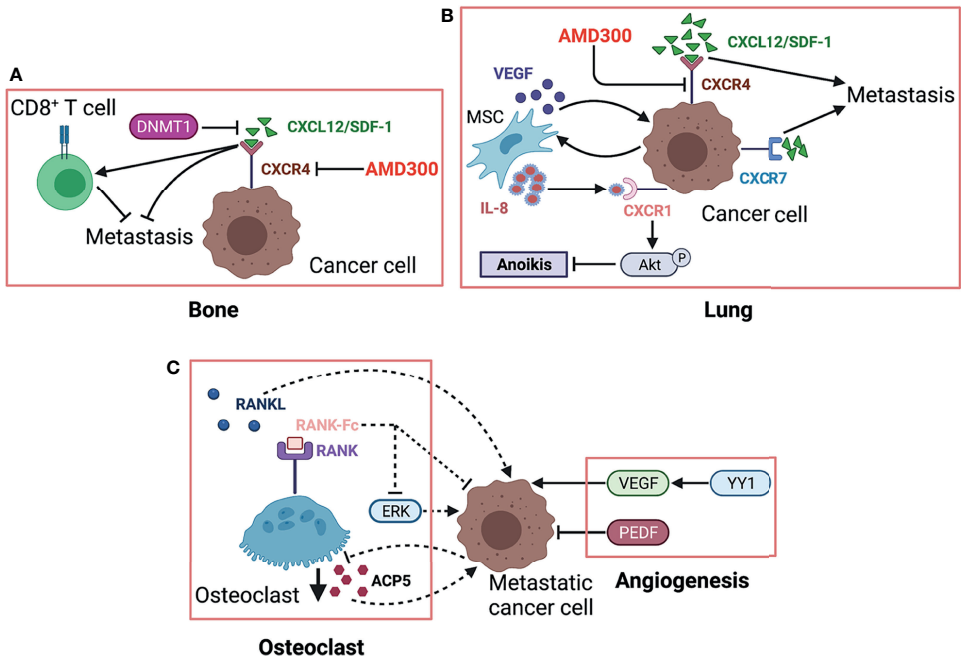


FIGURE 3 | The role of osteoclast and CXCR/CXCL axis in OS metastasis. **(A)** Highly expressed CXCL12. The CXCR4/CXCL12 (SDF-1) interaction is critical for OS metastasis in the lung, which is further strengthened by MSC *via* secreting VEGF. MSC-derived IL-8 induces OS cell anoikis resistance by activating CXCR1/Akt signaling. Another receptor CXCR7 expressed on OS cells promotes lung metastasis and enhances the malignancy activity of CXCR4. **(B)** RANK-Fc binds to RANK as a potent RANKL antagonist to inhibit osteoclast formation and activity, which can reduce OS metastasis, partly by suppressing ERK. Controversially, metastasis-competent OS cells induce loss of ACP5$^+$ osteoclasts, which in turn enhances metastasis. Herein, we used dotted lines to indicate this contradiction. VEGF exhibits prometastatic effects on OS cells while PEDF shows the opposite by regulating angiogenesis. **(C)** In primary bone site, OS epigenetically downregulates CXCL12 expression by DNMT1, impairs cytotoxic T-cell homing to the tumor site, and this chemokine gradient of CXCL12 drives the metastasis of OS cells to the lung. ACP5/TRAP, osteoclast-specific tartrate-resistant acid phosphatase 5; PEDF, pigment epithelium-derived factor; YY1, Yin Yang 1 protein; CXCL12, C–X–C motif chemokine 12; DNMT1, DNA methyltransferase 1; CXCR4, chemokine receptor 4; AMD3100, CXCR4 antagonist.

against transcription factor Sp7, TNFSF10 and TGF-β/Smad signaling. Anti-osteoclast drug like zoledronic acid could be synergistically used to enhance the therapy efficacy for OS progression (219). One publication has discussed the prospects of novel therapeutic strategy targeting osteoclast activity for OS (220). However, Munoz et al. suggested the tendency of mutual restrain between metastatic OS cells and osteoclasts and that metastasis-competent OS cells induce the loss of ACP5+ osteoclasts, which in turn facilitate metastasis (119). Notably, EVs derived from OS could suppress osteoclastogenesis and further enhance its metastasis (65). An *in vitro* study was performed to directly explore the reciprocal modulation between OS and osteoclastic cells by a co-culture system (221). More studies are needed to further clarify the exact role of osteoclast in OS metastasis. The interaction between osteoclasts and OS cells was displayed in **Figure 3C**.

Angiogenesis and Metastasis

Angiogenesis is an essential component for tumor growth and progression by supplying adequate blood and nutrients (75, 222). Additionally, these new blood vessels provide the principal route by which cancer cells exit the primary site and enter circulation (223). Angiogenesis is also required for tumor colonization at the site of metastasis. In OS metastasis, intensive investigation has explored the role of angiogenesis and interaction between proangiogenic and antiangiogenic factors, in order to develop potential optimum targets for antiangiogenic therapy (**Figure 3A**). A meta-analysis including nine articles revealed that VEGF is positively associated with tumor metastasis and a higher tumor grade (70). Two publications also suggested that angiopoietin-like protein 2 and YY1 accelerate metastasis *via* VEGF-mediated angiogenesis (112, 224). Furthermore, *VEGF* knockdown (73) restricts OS metastasis. Also, the blockade of VEGF receptor 2 (VEGFR2) by anlotinib, a tyrosine kinase inhibitor, results in metastasis suppression in a preclinical study (225). In contrast to VEGF-related angiogenesis, pigment epithelium-derived factor (PEDF) or PEDF-derived synthetic peptides both exhibited antimetastasis activity by inhibiting angiogenesis (77, 79, 81, 226). Antiangiogenesis therapy seems to be an appealing and attractive alternative strategy to manage OS metastasis. However, the safety and efficacy of antiangiogenic therapy has not been confirmed in clinical trials, more work is needed to achieve clinical transformation of this treatment. Current research progress and application limitation in antiangiogenesis therapy for OS can refer to other reviews (227, 228).

Metabolism and Metastasis

The metabolic program of primary tumor cells is quite different from that of metastatic cells in many ways such as nutritional availability, energy demand, oxygenation level, metabolites, and metabolic pathways, all of which participate in tumor metastasis (229). An advanced metabolomics has shed light on the study of biochemical status and reprogrammed metabolism during metastasis. Metabolomics covers a wide range of metabolites, mainly including sugars, amino acids, and fatty acids. Giang et al. have observed the Warburg effect in several human OS cell lines (230), a common phenomenon of aerobic glycolysis in cancer cells that means pyruvate is transformed into lactic acid even in the presence of oxygen (231). Further study in mice by Hua and colleagues found the dynamic metabolic reprogramming throughout tumor occurrence and progression (232). They identified a number of differentially expressed metabolic biomarkers in serum prior and postmetastasis. Their results suggested the metabolic reprogramming in OS metastasis, characterized by lowered carbohydrate and amino acid metabolism, while an elevated lipid metabolism. Moreover, the serum metabolic profile of lung metastasis is distinct from that of primary tumor. Mice developing lung metastasis have a higher level of lipid metabolites in serum compared with mice without metastasis (232). Consistently, a global analysis of lipidomic reveals the alteration of lipid profiles in metastatic OS cells (233). Previous studies using synvinolin (inhibitor of *de novo* cholesterol synthesis) observed metastasis reduction, which further supported a critical role of lipid metabolism in tumor metastasis (234, 235). The inositol pathway is significantly downregulated in highly metastatic OS cells (236). Functional study both in *ex vivo* lung culture and *in vivo* mouse model has indicated that the administration of inositol hexaphosphate, which will be converted to inositol once entering cells, can reduce lung metastasis by suppressing MAPK and PI3K signaling pathways.

HIF1-α expression is positively associated with metastasis while negatively with survival. Naggar et al. has shown that Y-box-binding protein 1 (YB-1) facilitates metastasis by direct translational activation of epithelial-to-mesenchymal transition (EMT) and HIF1-α, which then induces CXCR4 expression (194). Functionally, HIF1-α enhances invasion of OS cells in hypoxia *via* increasing VEGF-A (237). Another pathway, HIF1-α/CXCR4 is also proven to facilitate metastasis *in vitro* and *in vivo* (238). Additionally, HIF-1α binds to AP-1-binding motif within Cyr61 (also called CCN1) promoter and induces Cyr61 expression, which plays a prometastatic role in human melanoma cells under hypoxia (239). Hypoxia is one of the prominent features of many malignant tumors and also provides an opportunity to develop agents that target hypoxic region. For instance, hypoxia-activated prodrug TH-302 can reduce OS metastasis as a single agent or in combination with chemotherapy (240). The high expression of Cyr61 indicates poor survival in OS patients (91) and promotes metastasis *via* activating PI-3K/Akt/GSK3β, IGF1/IGFR signaling pathways, facilitating angiogenesis characterized by an increase in VEGF, FGF2, PECAM, and a decrease in TSP-1 and SPARC (93) and promoting EMT-like process (95). Of note, WW domain containing oxidoreductase (*WWOX*) as tumor suppressor has been revealed to maintain mitochondrial respiration and attenuate Warburg effect by inhibiting HIF1-α (241) and c-Jun (242) by physical interaction with them. Recently, a research group further found that WWOX inhibits OS metastasis *in vitro* and *in vivo* through downregulation of RUNX2 (243). The suppression of c-Jun activity can inhibit metastasis by increasing apoptosis (85, 89) and chemosensitivity in OS cells (87). Tang and colleagues demonstrated that cadherin-4 (CDH4) overexpression would activate c-Jun *via* the JNK pathway (83). In addition to c-Jun, proteins from several families like c-Fos, ATF, and MAF, can form transcriptional complex AP-1, and their activation may be one of the mechanisms underlying metastasis (244). Leaner et al. have shown a higher activity of AP-1 in highly metastatic OS cells, compared with low metastatic counterparts (245). In terms of OS, AP-1 promotes metastasis by upregulating podoplanin and TGF-β (246). Another study also reported that FGFR1 silence, a downstream target of c-Fos/AP-1 complex, could significantly reduce lung metastasis (247). The growth and progression of malignant tumors largely rely on glycolysis for energy, resulting in an acidic tumor microenvironment, which may in turn affect the biological behaviors of tumor cells. Several studies have investigated how OS cells adapt to acidic microenvironment. Brief exposure of OS cells to acidic condition results in cell death, whereas prolonged exposure reverses cell death, and even fosters tumor invasiveness (40). The adaption of OS cells to acidosis leads to a metabolic reprogramming with epigenetic stability (248). The underlying mechanism has been further explored, by which acid microenvironment activates a stress-regulated switch to support cell survive. Studies found that cIAP proteins and NF-κB pathway are activated in OS cells in response to acid tumor microenvironment (249, 250). Metabolism-related regulatory networks are shown in **Figure 4**.

As mentioned above, redox stress is a metabolic challenge in lung microenvironment for tumor cells. Studies shown that the excess reactive oxidative species (251, 252) and reactive nitrogen species (253, 254), both impair mitochondrial function. In order to overcome such oxidative pressures, metastatic OS cells trigger their antioxidant response by upregulating redox-related enzymes or glutathione-related metabolic pathways (255, 256). However, the previous mentioned study by Hua et al. reported the contradictory result that glutathione pathway is suppressed at the metastasis phase (232). More studies are urgently needed to further elucidate the exact role of metabolism especially glucose metabolism in tumor cells during dynamic metastatic process. Thereby, the exploration of metabolism reprogramming and metabolic vulnerability may provide novel targets for treating metastasis.

Immunity and Metastasis

It is well known that immune system both innate and adaptive immunity plays a key role in tumorigenesis and progression, and even to a large extent determines the fate of tumor cells. With the advance in basic and preclinical research, increasing immune-based therapies are being approved for clinical use in various

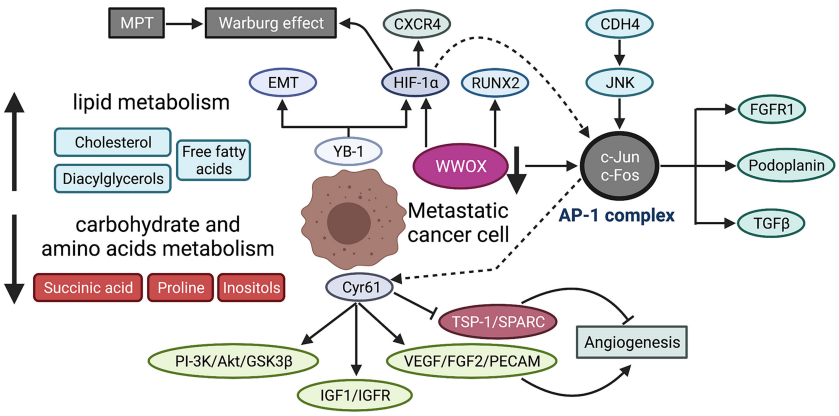


FIGURE 4 | Metabolic reprogramming during OS metastasis. MPT promotes Warburg effect in OS cells by suppressing mitochondrial function. The serum metabolic profile of lung metastasis shows lowered carbohydrate and amino acid metabolism but an elevated lipid metabolism. YB-1 contributes to metastasis by translational activation of EMT and HIF-1α, which then induces CXCR4 expression. Cyr61 enhances the metastatic potential of OS cells through multiple signaling pathways, including PI-3K/Akt/GSK3β, IGF1/IGFR, and angiogenesis-associated signaling (increased VEGF, FGF2, PECAM and reduced TSP-1, SPARC). WWOX maintains mitochondrial respiration and inhibits Warburg effect by physical interaction with HIF-1α. WWOX also suppresses c-Jun activity by physical association while CDH4 overexpression activates c-Jun *via* the JNK pathway. AP-1 is a transcriptional complex, mainly composed of c-Jun and c-Fos, which promotes metastatic potential by upregulating several downstream effectors, including FGFR1, podoplanin, and TGFβ. The dotted lines indicate the mechanistic study is performed in melanoma cells, that is, HIF-1α interacts with AP-1, which then binds to AP-1-binding motif within the Cyr61 promoter and induces Cyr61 expression. MPT, mitochondrial permeability transition; YB-1, Y-box binding protein 1; EMT, epithelial-to-mesenchymal transition; Cyr61, cysteine-rich protein 61; IGF-1, insulin-like growth factor 1; FGF2, fibroblast growth factor 2; PECAM, platelet endothelial cell adhesion molecule; TSP-1, thrombospondin-1; SPARC, secreted protein acidic and rich in cysteine; WWOX, WW domain-containing oxidoreductase; CDH4, cadherin-4; AP-1, activating protein-1; FGFR1, fibroblast growth factor receptor 1.

cancers, which yield encouraging outcomes in those patients who are resistant to conventional treatment. Nevertheless, the application of immunotherapy in metastatic OS is far from satisfactory to date. Recently, a series of studies identified several immune cells, including innate immunocytes (e.g., macrophages and dendritic cells) (257–259) and adaptive immunocytes (e.g., T lymphocytes) (260), all of which putatively participate in immune response during OS metastatic progression (**Figure 5**). The number, function, state of immune cells, and their interactions with each other collectively determine whether they promote or inhibit metastasis. Researchers have also recognized some immunocytes as diagnostic or prognostic biomarkers. For instance, the increase of M2-polarized tumor-associated macrophages (TAMs) maintains OS stemness and metastatic property (261). In addition, TAMs activated by mifamurtide (262) or M2-type macrophages suppressed by all-trans retinoic acid (ATRA) (263, 264) both can inhibit tumorigenicity and progression of OS cells. However, the inconsistent results among studies are available, which require more research to further elucidate the dynamic and complex role of TAMs in OS. In addition to TAMs, tumor-infiltrating lymphocytes (TILs) within OS microenvironment also affect OS progression (261, 265). The increased $CD8^+$ TILs as well as the high ratio of $CD8^+/FOXP3^+$ TILs both predict a better prognosis in OS patients (266, 267). However, the higher density of TILs is found to be within metastatic sites compared with primary tumor, which constitutes a special immune niche (82, 267). It should be noted that the interaction between PD-L1 (expressed in metastatic OS cells) and PD-1 (expressed in tumor-infiltrating cytotoxic T lymphocytes) limits antitumor function of T cells and thus promotes OS metastasis by evading immune surveillance. The dynamic and intricate regulation of immune system seems to be decisive for metastasis with respect to synthesis of immune factors, spatiotemporal expression of surface markers and phenotypic transition of immunocytes, and their interactions with surrounding compositions. With increasing understanding of immunomodulatory mechanism underlying OS metastasis, researchers are constantly exploring the key immune checkpoints to develop potential immunotherapy strategies for OS with the aim of improving prognosis. A recent review has discussed the mechanisms and status of immunotherapy for OS (268).

NONCODING RNA

Noncoding RNAs are a large group of RNAs characterized by the lack of ability to encode proteins, which can be divided into regulatory and housekeeping noncoding RNAs (269). Here, we focused on the role of the former in OS metastasis, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs). We further outlined the biological function of these noncoding RNAs in OS metastasis and the underlying molecular mechanism. **Table 2** summarizes the noncoding RNAs that have been validated *in vivo*.

MicroRNA

MiRNAs contain 18–25 bases and most of them fell within 21–23 bases. Typically, miRNAs bind to 3′untranslated region (3′UTR) of mRNAs by complementarily pairing to destabilize mRNAs or

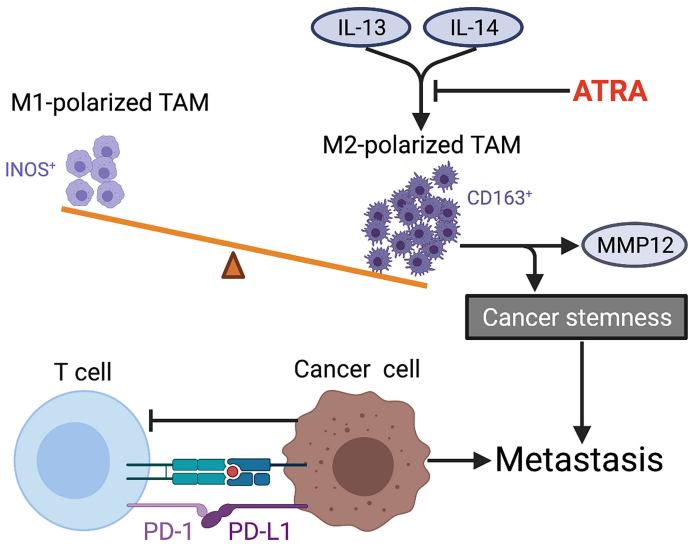


FIGURE 5 | Tumor immune microenvironment characteristics within OS metastasis. The imbalance of M1 (INOS$^+$)/M2 (CD163$^+$)-polarized TAMs in favor of M2 subtype is observed in metastatic OS. ATRA suppresses IL-13-induced secretion of MMP12 from M2-polarized macrophages and also weakens cancer stemness by preventing M2 polarization of TAMs in OS. The interaction between PD-L1 (expressed in metastatic OS cells) and PD-1 (expressed in tumor-infiltrating CTLs) limits antitumor function of T cells and thus promotes OS metastasis by evading immune surveillance. TAMs, tumor-associated macrophages; ATRA, all-trans retinoic acid; PD-L1, programmed death ligand 1; PD-1, programmed death receptor-1; CTLs, cytotoxic T lymphocytes.

block translation and thus reduce their expressions (349–351). A single miRNA can target multiple mRNAs and vice versa, one mRNA can be regulated by various miRNAs. Thereby, miRNAs may indirectly affect various biological processes such as proliferation, differentiation, apoptosis, and angiogenesis. The unique signatures of miRNA expression profile have been studied in OS metastasis as diagnostic and/or prognostic biomarkers. More importantly, an enormous growing body of studies have explored potential mechanisms, by which miRNAs participate in OS metastasis.

A minority of the miRNAs that we retrieved from literature have been demonstrated for their effects on OS metastasis *in vivo*. Also, only few miRNAs show prometastatic activity in OS, while the majority of miRNAs inhibit metastasis. A study reported that miR-195 suppressed metastatic potential by targeting FASN (352). However, other studies found a high level of miR-195 in serum from OS patients (353, 354). Thus, a single miRNA might play a dual role in different stages of OS progression or the seemingly contradictory results are probably caused by small number of participants. Further studies are needed to confirm the exact function of such miRNAs like miR-195. Whether miRNAs promote or inhibit metastasis depends on proteins they target. Intriguingly, miR-27a* is the first passenger miRNA strand and shows prometastatic activity in OS cells, which is encoded by *MIR27* and directly connects with CBFA2T3, the same as miR-27a (355). Furthermore, the injection of OS cells expressing miR-27a to mice can generate metastatic nodules within lung and bone *in vivo* (356, 357). In turn, miRNAs are also regulated by metastasis-associated genes such as *MYC*, *TP53*, and *TGFB1*. The epigenetic modulation affects the miRNA expression as well. One research team found that apurinic/apyrimidinic endodeoxyribonuclease 1 (*APEX1*) participated in multiple biological processes of OS by shifting miRNA expression profiles (358). Some drugs used to treat OS-like epirubicin (359) and diallyltrisulfide (360) can also alter miRNA expression profiles.

Unfortunately, miRNA-mediated therapy still remains the first phase of clinical trial at best despite so much basic and preclinical research. The main issue needed to be solved is how to deliver miRNAs efficiently and precisely to the sites of interest. Current methods generally show the following deficiencies, including low transfection efficiency, rapid degradation, and abnormal accumulation in nonspecific tissues and organs. Using hyaluronic acid-associated liposomes as carrier may accelerate the entry of miRNAs into cells and prevent them from degradation (361). An alternative approach is to apply viral vectors with hairpin molecules that will be processed into mature miRNAs (362).

Long Noncoding RNA

LncRNAs are a large group of noncoding RNAs with more than 200 nucleotides and emerging evidence demonstrates its crucial role in multiple biological processes, particularly in tumorigenesis (363, 364). Briefly, lncRNAs in nucleus can regulate gene expression both at genetic and epigenetic levels by affecting transcription factors and chromatin-modifying complexes to bind specific gene loci. In cytoplasm, lncRNAs modulate the stability and translation activity of mRNAs either directly or indirectly. The majority of lncRNAs included in our study compete with miRNAs to bind target mRNAs as sponges. Recently, lncRNAs have engaged much attention in the field of tumor aggressiveness, including migration, invasion, and

TABLE 2 | Summary of noncoding RNAs involved in osteosarcoma metastasis.

Author	Noncoding RNA	Pro/anti	Related genes or pathways
microRNA			
Gao et al. (270)	miR-17	Pro	PTEN↓
Ding et al. (271)	miR-18a	Anti	MED27, Akt↓
Sun et al. (272)	miR-19	Pro	SOCS6↓; JAK2/STAT3↑
Xin et al. (273)	miR-22	Anti	ACLY, lipogenesis↓
He et al. (274)	miR-23a	Anti	RUNX2, CXCL12↓
Chen et al. (275)	miR-25	Anti	SOX4, EMT↓
Lu et al. (276)	miR-26a	Anti	Jagged1/Notch, ALDH, stemness↓
Zhang et al. (277)	miR-30a	Anti	RUNX2↓
Tao et al. (278)	miR-30a-5p	Anti	FOXD1↓
Zhao et al. (279)	miR-34a	Anti	SIRT1, c-MET, CDK6↓
Liu et al. (280)	miR-92a	Anti	Notch1↓
Yu et al. (281)	miR-124	Anti	TGF-β/Akt/GSK-3β/SNAIL-1↓
Liu et al. (282)	miR-125b	Anti	STAT3↓
Bao et al. (283)	miR-134	Anti	–
Li et al. (284)	miR-137	Anti	FXYD6↓
Shi et al. (285)	miR-139-5p	Anti	DNMT1↓
Gu et al. (286)	miR-140	Anti	–
Xiao et al. (287)	miR-140	Anti	HDAC4↓
Wang et al. (288)	miR-144	Anti	ROCK1, ROCK2↓
Li et al. (289)	miR-145	Anti	CDK6↓
Yang et al. (290)	miR-148a	Anti	ROCK1↓
Zhou et al. (291)	miR-154	Anti	Wnt5a↓
Jiang et al. (292)	miR-181a	Pro	PTEN↓
Zhang et al. (293)	miR-186-5p	Anti	FOXK1, EMT↓
Pan et al. (294)	miR–188	Anti	SOX4↓
Pu et al. (295)	miR-193a-3p; miR-193a-5p	Anti	Rab27B, SRR↓
Li et al. (296)	miR-204-5p	Anti	EBF2↓
Jiang et al. (297)	miR-208b	Anti	ROR2↓
Liu et al. (298)	miR-210	Pro	–
Luo et al. (299)	miR-212	Anti	SOX4↓
Xu et al. (300)	miR-214	Pro	LZTS1↓
Sun et al. (301)	miR-217	Anti	–
Jiang et al. (302)	miR-329	Anti	Rab10↓
He et al. (303)	miR-363	Anti	PDZD2, EMT↓
Xu et al. (304)	miR-372-3p	Anti	FXYD6↓
Li et al. (305)	miR-379	Anti	PDK1↓
Zhao et al. (306)	miR-410	Anti	VEGF↓
Yang et al. (307)	miR-425-5p	Anti	MALAT1, TUG1, Wnt/β-catenin↓
Yuan et al. (308)	miR-451	Anti	–
Yuan et al. (309)	miR–494	Anti	CDK6↓
Qi et al. (310)	miR–496	Anti	eIF4E↓
Pang et al. (311)	miR–497	Anti	–
Cai et al. (312)	miR–590–5p	Anti	KLF5↓
Liu et al. (313)	miR–598	Anti	–
Ma et al. (314)	miR–603	Pro	BRCC2↓
Wang et al. (315)	miR–643	Anti	ZEB1↓
Zhang et al. (316)	miR–663a	Anti	ZBTB7A↓; LncRNA GAS5↑
Liu et al. (317)	miR–873	Anti	HOXA9, Wnt/β–catenin↓
Tanushree et al. (318)	miR–874	Anti	CCNE1↓
Zhong et al. (319)	miR–1270	Anti	–
Yuan et al. (320)	miR–1908	Anti	PTEN↓
Long noncoding RNA			
Shi et al. (321)	AFAP1-AS1	Pro	RhoC/ROCK1/p38MAPK/Twist1↑
Li et al. (322)	AFAP1-AS1	Pro	miR–4695–5p↓ TCF4–Wnt/β–catenin↑
Lu et al. (323)	CASC2	Anti	–
Zhao et al. (324)	EPIC1	Anti	MEF2D↓
Zhu et al. (325)	FOXF1-AS1	Pro	FOXF1/MMP2/9↑
Sun et al. (326)	FGFR3-AS1	Pro	FGFR3↑
Ren et al. (327)	FOXD2-AS1	Pro	EZH2, p21↓
Ye et al. (328)	GAS5	Anti	miR-221, EMT↓; ARHI↑
Qu et al. (329)	HOXD-AS1	Pro	STAT3, MMP2↑
Wang et al. (330)	HOTAIR	Pro	MMP2/9↑

(Continued)

TABLE 2 | Continued

Author	Noncoding RNA	Pro/anti	Related genes or pathways
Gu et al. (331)	LINC00858	Pro	miR-139↓; CDK14↑
Zhang et al. (332)	LINC01116	Pro	miR-520a-3p↓; IL6R, JAK/STAT↑
Han et al. (333)	LUCAT1	Pro	miR-200c↓; ABCB1↑
Chen et al. (334)	MALAT1	Pro	miR-129-5p↓; RET-PI3K/Akt, stemness↑
Dong et al. (335)	Mal. AT1	Pro	p85α, PI3K/Akt, MMP9, PCNA↑
Duan et al. (336)	MALAT1	Pro	miR–34a↓; cyclin D1↑
Li et al. (337)	miR210HG	Pro	miR-503↓; EMT, N-cadherin, vimentin↑
Hu et al. (338)	NEAT1	Pro	miR-34c↓
Ye et al. (339)	NNT-AS1	Pro	–
Zhu et al. (340)	ODRUL	Pro	miR-3182↓; MMP2↑
Wang et al. (341)	SNHG1	Pro	miR-326↓; NOB1
Jiang et al. (342)	SNHG1	Pro	miR-577↓; WNT2B/Wnt/β-catenin, EMT↑
Deng et al. (343)	SNHG7	Pro	miR-34a↓; TGF-β, SMAD4, EMT↑
Yang et al. (344)	TP73-AS1	Pro	miR-142↓; Rac1↑
Wang et al. (345)	TUG1	Pro	miR-153↓
Yang et al. (346)	XIST	Pro	miR-195-5p↓; YAP↑
Zhang et al. (347)	XIST	Anti[a]	miR-21-5p, EMT↓; PDCD4↑
Circular RNAs			
Liu et al. (348)	CircFAT1	Pro	miR-375↓; YAP 1↑

Studies using animal models of osteosarcoma metastasis are included.

Pro, the target gene promotes metastasis; anti, the target gene inhibits metastasis; "↑" upregulation; "↓" downregulation.

[a]*The results in this article is contrary to that of the previous one.*

metastasis. Unlike miRNAs, most of lncRNAs involved in OS are positively associated with metastatic property. We outlined those lncRNAs that have been widely studied in OS metastasis and confirmed *in vivo*.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also named noncoding nuclear-enriched abundant transcript 2 (NEAT2), is located in chromosome 11q13 with a length of 8.7kb (365). The MALAT1 overexpression is closely correlated with advanced clinical stage and distant metastasis in OS patients (366). Furthermore, a meta-analysis confirmed the predictive value of MALAT1 for metastasis and poor prognosis in six different types of cancer, including OS (367). Consistent with this result, another meta-analysis only including OS also showed MALAT1 as a valuable biomarker for prognosis (368). MALAT1 knockdown inhibits OS metastasis *in vitro* and *in vivo* by affecting various downstream pathways, such as downregulation of PI3K/Akt/MMP-9 signaling pathway (334, 335) and upregulation of E-cadherin and several miRNAs (336, 369). Also, high dose of 17β-estradiol inhibits metastatic potential of OS cells by suppressing MALAT1 while low dose presents the opposite effect (370, 371). It is noteworthy that the most widely studied lncRNAs is the large family of small nucleolar RNA host gene (SNHG), of which all members are considered to promote OS progression and metastasis by sponging various miRNAs. SNHG1, for example, activates Nin one binding protein (NOB1) by sponging miRNA-326 and thus promotes migration/invasion in OS cells and metastasis in murine models (341). Consistently, additional research further shows that SNHG1 is positively related to advanced clinical stage, distant metastasis, and poor survival of OS patients. In addition, SNHG1 facilitates cell proliferation, migration, and invasion *via* activating PI3K/AKT and Wnt/β-catenin pathways by sequestrating various miRNAs (341, 342, 372). Besides, SNHG12 promotes metastasis by increasing angiomotin (373), Notch2 (374) and IGF1R (375) through sponging miRNA-195-5p. There are two research groups that independently demonstrated the role of lncRNA DANCR in OS progression and possible underlying mechanisms. As a competitive endogenous lncRNA, DANCR either increases rho-associated protein kinase 1 (ROCK1) through decoying miRNA-335-5p and miRNA-1972 (376), or enhances AXL/PI3K/Akt signaling pathway by sponging miRNA-33a-5p (377), and thereby promotes OS metastasis.

Circular RNA

CircRNAs are a newly identified class of endogenous noncoding RNAs characterized by closed-loop structures. Unlike typical linear RNA, circRNAs do not contain 5′-3′ polarity or polyadenylated tail and is thus resistant to enzyme degradation (378, 379). Similar to lncRNAs, circRNAs can competitively bind miRNAs as sponges to indirectly regulate gene expression. Currently, increasing evidence indicates that circRNAs have an important role in many biological behaviors and diseases, particularly in tumorigenesis and tumor progression. The dysregulation of some circRNAs has also been identified in OS metastatic process (380). Almost all circRNAs, like lncRNAs, act as sponges of miRNAs to disinhibit specific prometastatic pathways that normally are inhibited by miRNAs in OS. However, it is not always the case. It has been reported that low expression of circ-HIPK3 is observed in OS cell lines, tissues, and plasm and associated with lung metastasis (381). Overexpression of circ-HIPK3 can inhibit proliferation, migration, and invasion in OS cells. These findings indicate that circ-HIPK3 may have great value in clinical practice as a tumor suppressor. Intriguingly, circ-HIPK3 plays the opposite role in tumor occurrence and progression in other tumors, which strongly suggests that whether a single circRNA acts as an oncogene or a tumor suppressor gene depends on tumor types and which miRNA it sponges (382–386). In addition, circRNAs hsa_circ_0002052 and

circ-ITCH are also found to be downregulated in OS cell lines and tissues. Functional studies further confirm that their overexpression can suppress OS cell progressiveness by inhibiting Wnt/β-catenin pathway *via* targeting miRNA-1205/APC2 axis (387), and by suppressing PTEN/PI3K/AKT and Sp1 pathways *via* sponging miRNA-22 (388), respectively. So far, the knowledge and research on circRNAs in OS metastasis are much less than that of miRNAs or lncRNAs. Nevertheless, the special annular structure gives circRNAs unique advantages with stronger stability and higher abundance.

In addition to tumor tissues, collecting noncoding RNAs from blood is convenient in clinical practice. Notably, a number of studies have reported that exosomes could carry noncoding RNAs (212, 213) and even transport them into OS cells (210), thus foster OS metastasis. With the deeper understanding of the relationship between noncoding RNAs and clinicalpathological characteristics, noncoding RNAs will help early diagnosis of metastasis, monitoring of treatment, and prediction of prognosis in OS patients. Although there is no therapy targeting noncoding RNAs used for OS yet, further research will make significant progress to develop novel therapeutic strategies and improve outcomes for OS patients with metastasis.

DISCUSSION

In conclusion, metastasis is the most important factor resulting in treatment failure and the leading cause of death in OS patients. The 5-year survival rate for patients with metastasis remains only about 20% despite the use of aggressive surgery and intensive chemotherapy. Approximately 20% of patients present detectable metastasis at their first visit to hospital. However, almost all patients with localized disease are assumed to have micrometastasis already and nearly half of them will progress to clinical metastasis.

Although there have been great advances in the research of OS metastasis in the past few decades, the underlying mechanism is not yet clearly elucidated. Generally, the formation of new blood vessels by endothelial cells is a prerequisite for both primary and metastatic tumor growth. For OS, publications suggested that OS cell could facilitate endothelial cell proliferation (389) and several reagents have been tested to suppress angiogenesis of endothelial cells by directly acting on OS cells (390, 391). Although seemingly inconsistent with tumor angiogenesis, OS cells induce contact-dependent endothelial apoptosis, which may contribute to tumor invasion across vascular barrier during metastasis (29). Therefore, the role of endothelial cells and the interaction between them and OS cells are complex, perhaps playing a dual role, or changing dynamically with the stage and site of OS metastasis.

Lung is the most common site of OS metastasis; however, the underlying mechanism of this apparent organotropism remains to be elucidated. Recently, research found that secreted extracellular vesicles, specifically exosomes, would prepopulate in a particular organ, making it more suitable for tumor metastasis. Metastatic organotropism is one of the prominent characteristics of OS, and more investigations are necessary to reveal the potential mechanisms. In addition, novel therapy targeting those molecules that direct OS cells to lung may yield encouraging outcomes in OS patients. Also, immunotherapy has occupied an important position in the field of tumor therapy. However, no immunotherapy has made a breakthrough in metastatic osteosarcoma so far. The role of immune system in OS development and progression may differ from other tumors. Efforts should be made to explore the unique immune niche of OS metastasis, supporting performance of clinical transformation. In addition to external environmental and genetic factors, physical stimuli and modulation significantly influence OS metastatic process. The interaction of metastatic OS cells with their microenvironment, where they encounter dynamic mechanical forces, definitely affects OS cell invasiveness and to some extent determines the preferred metastatic site. However, it is not fully understood how OS cells specifically respond and adapt to their mechanical surroundings. Besides, the substrate stiffness and fluid flow shear stress may also modify the response of OS to therapy. Thus, the research on the fluid flow and/or substrate pressure can provide a novel consideration for developing anti-metastasis therapy.

Herein, we summarize the key regulatory molecules, signaling pathways, and dysregulated noncoding RNAs during metastatic process and hope to reveal potential druggable targets. The innovative technologies have ushered OS metastasis research into new era, such as multi-omics, liquid biopsy, tissue engineering, bioluminescent imaging, and so on. The OS research community should create biological banks and online databases to store and share precious specimens and relevant research results. Bioinformatics is an effective approach to reanalyze the raw data and explore the most valuable information. Moreover, there is a complex regulatory network between microenvironment, angiogenesis, osteoclast, metabolism, immunity, drug resistance, and OS metastasis. Based on emerging technologies and methods, it is expected to depict this network and expand our understanding of metastasis biology.

The standard drug development platform and evaluation system are necessary for clinical translation of promising drugs specifically in rare diseases like OS. As such, it is essential to establish various cell lines and biological models including animal models and bioengineering models that will facilitate research on metastasis. The models especially canine model that faithfully recapitulate metastasis development as in humans are prerequisites for clinical trials of candidate drugs. In addition, we should pay more attention to the effect of drugs on micrometastasis since some drugs uniquely reduce subclinical lesions (392–394). The novel drug delivery strategy and routes may allow conventional drugs to achieve unexpected efficiency while decreasing systemic toxicity (395–397).

The early diagnosis of metastasis and detection of circulating OS cells or micrometastasis will be the hotspots of future research and lead to breakthrough in improving survival of metastatic patients. A sensitive immunomagnetic detection assay has been successfully used to detect micrometastases from bone marrow and peripheral blood (398, 399). Molecular imaging with specific molecular probes are also attempted to track OS cells, such as

CXCR4-targeted near-infrared fluorescence imaging (400) and ssDNA aptamer LP-16 targeting metastatic OS cells (401), which both allow detection of OS microlesions.

Drug therapy may be the only option to completely eliminate micrometastasis or inoperable lesions. The increasing studies have identified more and more biomarkers that are of predictive value for metastasis and prognosis in OS patients. However, individualized comprehensive molecular profiling of OS patients has not significantly changed the therapeutic prospects of advanced osteosarcoma. Therefore, more work is needed to better classify patients and further propel personalized management for specific patients. In short, only overcoming metastasis can effectively improve the survival of patients with osteosarcoma.

AUTHOR CONTRIBUTIONS

The manuscript was drafted by GS. YG created the diagrams. YY was the guarantor of the entire manuscript for designing and supervising the entire study. HW checked and approved it. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant No. 51537004).

REFERENCES

1. Bielack S, Jurgens H, Jundt G, Kevric M, Kuhne T, Reichardt P, et al. Osteosarcoma: The COSS Experience. *Cancer Treat Res* (2009) 152:289–308. doi: 10.1007/978-1-4419-0284-9_15
2. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma Incidence and Survival Rates From 1973 to 2004: Data From the Surveillance, Epidemiology, and End Results Program. *Cancer* (2009) 115(7):1531–43. doi: 10.1002/cncr.24121
3. Whelan J, McTiernan A, Cooper N, Wong YK, Francis M, Vernon S, et al. Incidence and Survival of Malignant Bone Sarcomas in England 1979-2007. *Int J Cancer* (2012) 131(4):E508–17. doi: 10.1002/ijc.26426
4. Stiller CA, Craft AW, Corazziari I, Group EW. Survival of Children With Bone Sarcoma in Europe Since 1978: Results From the EUROCARE Study. *Eur J Cancer* (2001) 37(6):760–6. doi: 10.1016/s0959-8049(01)00004-1
5. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic Factors in High-Grade Osteosarcoma of the Extremities or Trunk: An Analysis of 1,702 Patients Treated on Neoadjuvant Cooperative Osteosarcoma Study Group Protocols. *J Clin Oncol* (2002) 20 (3):776–90. doi: 10.1200/JCO.2002.20.3.776
6. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary Metastatic Osteosarcoma: Presentation and Outcome of Patients Treated on Neoadjuvant Cooperative Osteosarcoma Study Group Protocols. *J Clin Oncol* (2003) 21(10):2011–8. doi: 10.1200/JCO.2003.08.132
7. Mialou V, Philip T, Kalifa C, Perol D, Gentet JC, Marec-Berard P, et al. Metastatic Osteosarcoma at Diagnosis: Prognostic Factors and Long-Term Outcome–the French Pediatric Experience. *Cancer* (2005) 104(5):1100–9. doi: 10.1002/cncr.21263
8. Kaste SC, Pratt CB, Cain AM, Jones-Wallace DJ, Rao BN. Metastases Detected at the Time of Diagnosis of Primary Pediatric Extremity Osteosarcoma at Diagnosis: Imaging Features. *Cancer* (1999) 86(8):1602–8. doi: 10.1002/(sici)1097-0142(19991015)86:8<1602::aid-cncr31>3.0.co;2-r
9. Harting MT, Blakely ML. Management of Osteosarcoma Pulmonary Metastases. *Semin Pediatr Surg* (2006) 15(1):25–9. doi: 10.1053/j.sempedsurg.2005.11.005
10. Bacci G, Rocca M, Salone M, Balladelli A, Ferrari S, Palmerini E, et al. High Grade Osteosarcoma of the Extremities With Lung Metastases at Presentation: Treatment With Neoadjuvant Chemotherapy and Simultaneous Resection of Primary and Metastatic Lesions. *J Surg Oncol* (2008) 98(6):415–20. doi: 10.1002/jso.21140
11. Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. *J Clin Oncol* (2015) 33 (27):3029–35. doi: 10.1200/JCO.2014.59.4895
12. Gorlick R, Anderson P, Andrulis I, Arndt C, Beardsley GP, Bernstein M, et al. Biology of Childhood Osteogenic Sarcoma and Potential Targets for Therapeutic Development: Meeting Summary. *Clin Cancer Res* (2003) 9 (15):5442–53.
13. PosthumaDeBoer J, Witlox MA, Kaspers GJ, van Royen BJ. Molecular Alterations as Target for Therapy in Metastatic Osteosarcoma: A Review of Literature. *Clin Exp Metastasis* (2011) 28(5):493–503. doi: 10.1007/s10585-011-9384-x
14. Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, et al. Metastasis-Associated Differences in Gene Expression in a Murine Model of Osteosarcoma. *Cancer Res* (2001) 61(9):3750–9.
15. Hynes RO. Metastatic Potential: Generic Predisposition of the Primary Tumor or Rare, Metastatic Variants-or Both? *Cell* (2003) 113(7):821–3. doi: 10.1016/s0092-8674(03)00468-9
16. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, et al. The Membrane-Cytoskeleton Linker Ezrin is Necessary for Osteosarcoma Metastasis. *Nat Med* (2004) 10(2):182–6. doi: 10.1038/nm982
17. Zucchini C, Rocchi A, Manara MC, De Sanctis P, Capanni C, Bianchini M, et al. Apoptotic Genes as Potential Markers of Metastatic Phenotype in Human Osteosarcoma Cell Lines. *Int J Oncol* (2008) 32(1):17–31. doi: 10.3892/ijo.32.1.17
18. Weiss L. Metastatic Inefficiency. *Adv Cancer Res* (1990) 54:159–211. doi: 10.1016/s0065-230x(08)60811-8
19. Cameron MD, Schmidt EE, Kerkvliet N, Nadkarni KV, Morris VL, Groom AC, et al. Temporal Progression of Metastasis in Lung: Cell Survival, Dormancy, and Location Dependence of Metastatic Inefficiency. *Cancer Res* (2000) 60(9):2541–6.
20. Tsuru A, Setoguchi T, Matsunoshita Y, Nagao-Kitamoto H, Nagano S, Yokouchi M, et al. Hairy/enhancer-Of-Split Related With YRPW Motif Protein 1 Promotes Osteosarcoma Metastasis *via* Matrix Metallopeptidase 9 Expression. *Br J Cancer* (2015) 112(7):1232–40. doi: 10.1038/bjc.2015.84
21. Liu JF, Chen PC, Chang TM, Hou CH. Thrombospondin-2 Stimulates MMP-9 Production and Promotes Osteosarcoma Metastasis *via* the PLC, PKC, C-Src and NF-kappaB Activation. *J Cell Mol Med* (2020) 24 (21):12826–39. doi: 10.1111/jcmm.15874
22. Osaki M, Takeshita F, Sugimoto Y, Kosaka N, Yamamoto Y, Yoshioka Y, et al. MicroRNA-143 Regulates Human Osteosarcoma Metastasis by Regulating Matrix Metalloprotease-13 Expression. *Mol Ther* (2011) 19 (6):1123–30. doi: 10.1038/mt.2011.53
23. Hirahata M, Osaki M, Kanda Y, Sugimoto Y, Yoshioka Y, Kosaka N, et al. PAI-1, a Target Gene of miR-143, Regulates Invasion and Metastasis by Upregulating MMP-13 Expression of Human Osteosarcoma. *Cancer Med* (2016) 5(5):892–902. doi: 10.1002/cam4.651
24. Ingvarsen SZ, Gardsvoll H, van Putten S, Norregaard KS, Krigslund O, Meilstrup JA, et al. Tumor Cell MT1-MMP is Dispensable for Osteosarcoma Tumor Growth, Bone Degradation and Lung Metastasis. *Sci Rep* (2020) 10 (1):19138. doi: 10.1038/s41598-020-75995-6
25. Zhang M, Zhang J, Zhou Q. Elevated Expression of microRNA-328-3p Suppresses Aggressive Malignant Behaviors *via* Targeting Matrix Metalloprotease 16 in Osteosarcoma. *Onco Targets Ther* (2019) 12:2063–70. doi: 10.2147/OTT.S195022
26. Kane SE, Gottesman MM. The Role of Cathepsin L in Malignant Transformation. *Semin Cancer Biol* (1990) 1(2):127–36.
27. Han XG, Li Y, Mo HM, Li K, Lin D, Zhao CQ, et al. TIMP3 Regulates Osteosarcoma Cell Migration, Invasion, and Chemotherapeutic

Resistances. *Tumour Biol* (2016) 37(7):8857–67. doi: 10.1007/s13277-015-4757-4
28. Cheng HL, Hsieh MJ, Yang JS, Lin CW, Lue KH, Lu KH, et al. Nobiletin Inhibits Human Osteosarcoma Cells Metastasis by Blocking ERK and JNK-Mediated MMPs Expression. *Oncotarget* (2016) 7(23):35208–23. doi: 10.18632/oncotarget.9106
29. McEwen A, Emmanuel C, Medbury H, Leick A, Walker DM, Zoellner H. Induction of Contact-Dependent Endothelial Apoptosis by Osteosarcoma Cells Suggests a Role for Endothelial Cell Apoptosis in Blood-Borne Metastasis. *J Pathol* (2003) 201(3):395–403. doi: 10.1002/path.1457
30. Bian ZY, Fan QM, Li G, Xu WT, Tang TT. Human Mesenchymal Stem Cells Promote Growth of Osteosarcoma: Involvement of Interleukin-6 in the Interaction Between Human Mesenchymal Stem Cells and Saos-2. *Cancer Sci* (2010) 101(12):2554–60. doi: 10.1111/j.1349-7006.2010.01731.x
31. Xu WT, Bian ZY, Fan QM, Li G, Tang TT. Human Mesenchymal Stem Cells (hMSCs) Target Osteosarcoma and Promote Its Growth and Pulmonary Metastasis. *Cancer Lett* (2009) 281(1):32–41. doi: 10.1016/j.canlet.2009.02.022
32. Kawano M, Tanaka K, Itonaga I, Iwasaki T, Miyazaki M, Ikeda S, et al. Dendritic Cells Combined With Anti-GITR Antibody Produce Antitumor Effects in Osteosarcoma. *Oncol Rep* (2015) 34(4):1995–2001. doi: 10.3892/or.2015.4161
33. Kawano M, Itonaga I, Iwasaki T, Tsumura H. Enhancement of Antitumor Immunity by Combining Anti-Cytotoxic T Lymphocyte Antigen-4 Antibodies and Cryotreated Tumor Lysate-Pulsed Dendritic Cells in Murine Osteosarcoma. *Oncol Rep* (2013) 29(3):1001–6. doi: 10.3892/or.2013.2224
34. Kawano M, Itonaga I, Iwasaki T, Tsuchiya H, Tsumura H. Anti-TGF-Beta Antibody Combined With Dendritic Cells Produce Antitumor Effects in Osteosarcoma. *Clin Orthop Relat Res* (2012) 470(8):2288–94. doi: 10.1007/s11999-012-2299-2
35. Fernandez L, Valentin J, Zalacain M, Leung W, Patino-Garcia A, Perez-Martinez A. Activated and Expanded Natural Killer Cells Target Osteosarcoma Tumor Initiating Cells in an NKG2D-NKG2DL Dependent Manner. *Cancer Lett* (2015) 368(1):54–63. doi: 10.1016/j.canlet.2015.07.042
36. Norregaard KS, Jurgensen HJ, Gardsvoll H, Engelholm LH, Behrendt N, Soe K. Osteosarcoma and Metastasis Associated Bone Degradation-A Tale of Osteoclast and Malignant Cell Cooperativity. *Int J Mol Sci* 22(13):6865. doi: 10.3390/ijms22136865
37. Tan K, Goldstein D, Crowe P, Yang JL. Uncovering a Key to the Process of Metastasis in Human Cancers: A Review of Critical Regulators of Anoikis. *J Cancer Res Clin Oncol* (2013) 139(11):1795–805. doi: 10.1007/s00432-013-1482-5
38. Wirtz D, Konstantopoulos K, Searson PC. The Physics of Cancer: The Role of Physical Interactions and Mechanical Forces in Metastasis. *Nat Rev Cancer* (2011) 11(7):512–22. doi: 10.1038/nrc3080
39. Follain G, Herrmann D, Harlepp S, Hyenne V, Osmani N, Warren SC, et al. Fluids and Their Mechanics in Tumour Transit: Shaping Metastasis. *Nat Rev Cancer* (2020) 20(2):107–24. doi: 10.1038/s41568-019-0221-x
40. Matsubara T, Diresta GR, Kakunaga S, Li D, Healey JH. Additive Influence of Extracellular Ph, Oxygen Tension, and Pressure on Invasiveness and Survival of Human Osteosarcoma Cells. *Front Oncol* (2013) 3199:199. doi: 10.3389/fonc.2013.00199
41. Coughlin TR, Sana A, Voss K, Gadi A, Basu-Roy U, Curtin CM, et al. The Effect of Fluid Flow Shear Stress and Substrate Stiffness on Yes-Associated Protein (YAP) Activity and Osteogenesis in Murine Osteosarcoma Cells. *Cancers (Basel)* (2021) 13(13):3128. doi: 10.3390/cancers13133128
42. Zachos TA, Aiken SW, DiResta GR, Healey JH. Interstitial Fluid Pressure and Blood Flow in Canine Osteosarcoma and Other Tumors. *Clin Orthop Relat Res* (2001) 385):230–6. doi: 10.1097/00003086-200104000-00034
43. Nathan SS, DiResta GR, Casas-Ganem JE, Hoang BH, Sowers R, Yang R, et al. Elevated Physiologic Tumor Pressure Promotes Proliferation and Chemosensitivity in Human Osteosarcoma. *Clin Cancer Res* (2005) 11(6):2389–97. doi: 10.1158/1078-0432.CCR-04-2048
44. Fidler IJ. Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled With 125 I-5-Iodo-2'-Deoxyuridine. *J Natl Cancer Inst* (1970) 45(4):773–82.
45. Holmgren L, O'Reilly MS, Folkman J. Dormancy of Micrometastases: Balanced Proliferation and Apoptosis in the Presence of Angiogenesis Suppression. *Nat Med* (1995) 1(2):149–53. doi: 10.1038/nm0295-149
46. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, et al. Transcriptional Switch of Dormant Tumors to Fast-Growing Angiogenic Phenotype. *Cancer Res* (2009) 69(3):836–44. doi: 10.1158/0008-5472.CAN-08-2590
47. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of Endoplasmic Reticulum Stress-Induced Apoptosis. *EMBO Rep* (2006) 7(9):880–5. doi: 10.1038/sj.embor.7400779
48. Yan M, Ni J, Song D, Ding M, Huang J. Activation of Unfolded Protein Response Protects Osteosarcoma Cells From Cisplatin-Induced Apoptosis Through NF-kappaB Pathway. *Int J Clin Exp Pathol* (2015) 8(9):10204–15.
49. Chaiyawat P, Sungngam P, Teeyakasem P, Sirikaew N, Klangjorhor J, Settakorn J, et al. Protein Profiling of Osteosarcoma Tissue and Soft Callus Unveils Activation of the Unfolded Protein Response Pathway. *Int J Oncol* (2019) 54(5):1704–18. doi: 10.3892/ijo.2019.4737
50. Lizardo MM, Morrow JJ, Miller TE, Hong ES, Ren L, Mendoza A, et al. Upregulation of Glucose-Regulated Protein 78 in Metastatic Cancer Cells Is Necessary for Lung Metastasis Progression. *Neoplasia* (2016) 18(11):699–710. doi: 10.1016/j.neo.2016.09.001
51. Yarapureddy S, Abril J, Foote J, Kumar S, Asad O, Sharath V, et al. ATF6alpha Activation Enhances Survival Against Chemotherapy and Serves as a Prognostic Indicator in Osteosarcoma. *Neoplasia* (2019) 21(6):516–32. doi: 10.1016/j.neo.2019.02.004
52. Chambers AF, Groom AC, MacDonald IC. Dissemination and Growth of Cancer Cells in Metastatic Sites. *Nat Rev Cancer* (2002) 2(8):563–72. doi: 10.1038/nrc865
53. Weibel ER. Lung Morphometry: The Link Between Structure and Function. *Cell Tissue Res* (2017) 367(3):413–26. doi: 10.1007/s00441-016-2541-4
54. Liu Y, Cao X. Characteristics and Significance of the Pre-Metastatic Niche. *Cancer Cell* (2016) 30(5):668–81. doi: 10.1016/j.ccell.2016.09.011
55. Hood JL, San RS, Wickline SA. Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis. *Cancer Res* (2011) 71(11):3792–801. doi: 10.1158/0008-5472.CAN-10-4455
56. Zhang H, Deng T, Liu R, Bai M, Zhou L, Wang X, et al. Exosome-Delivered EGFR Regulates Liver Microenvironment to Promote Gastric Cancer Liver Metastasis. *Nat Commun* (2017) 8:15016. doi: 10.1038/ncomms15016
57. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic Cancer Exosomes Initiate Pre-Metastatic Niche Formation in the Liver. *Nat Cell Biol* (2015) 17(6):816–26. doi: 10.1038/ncb3169
58. Zhou CF, Ma J, Huang L, Yi HY, Zhang YM, Wu XG, et al. Cervical Squamous Cell Carcinoma-Secreted Exosomal miR-221-3p Promotes Lymphangiogenesis and Lymphatic Metastasis by Targeting VASH1. *Oncogene* (2019) 38(8):1256–68. doi: 10.1038/s41388-018-0511-x
59. Guo Y, Ji X, Liu J, Fan D, Zhou Q, Chen C, et al. Effects of Exosomes on Pre-Metastatic Niche Formation in Tumors. *Mol Cancer* (2019) 18(1):39. doi: 10.1186/s12943-019-0995-1
60. Wortzel I, Dror S, Kenific CM, Lyden D. Exosome-Mediated Metastasis: Communication From a Distance. *Dev Cell* (2019) 49(3):347–60. doi: 10.1016/j.devcel.2019.04.011
61. Chicon-Bosch M, Tirado OM. Exosomes in Bone Sarcomas: Key Players in Metastasis. *Cells* 9(1):241. doi: 10.3390/cells9010241
62. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour Exosome Integrins Determine Organotropic Metastasis. *Nature* (2015) 527(7578):329–35. doi: 10.1038/nature15756
63. Wang J, Zhang H, Sun X, Wang X, Ren T, Huang Y, et al. Exosomal PD-L1 and N-Cadherin Predict Pulmonary Metastasis Progression for Osteosarcoma Patients. *J Nanobiotechnol* (2020) 18(1):151. doi: 10.1186/s12951-020-00710-6
64. Zhang H, Wang J, Ren T, Huang Y, Yu Y, Chen C, et al. LncRNA CASC15 is Upregulated in Osteosarcoma Plasma Exosomes and CASC15 Knockdown Inhibits Osteosarcoma Progression by Regulating miR-338-3p/RAB14 Axis. *Onco Targets Ther* (2020) 13:12055–66. doi: 10.2147/OTT.S282053
65. Araki Y, Aiba H, Yoshida T, Yamamoto N, Hayashi K, Takeuchi A, et al. Osteosarcoma-Derived Small Extracellular Vesicles Enhance Tumor Metastasis and Suppress Osteoclastogenesis by miR-146a-5p. *Front Oncol* (2021) 11:667109. doi: 10.3389/fonc.2021.667109

66. Wang L, Wu J, Song S, Chen H, Hu Y, Xu B, et al. Plasma Exosome-Derived Sentrin SUMO-Specific Protease 1: A Prognostic Biomarker in Patients With Osteosarcoma. *Front Oncol* (2021) 11:625109. doi: 10.3389/fonc.2021.625109
67. Macklin R, Wang H, Loo D, Martin S, Cumming A, Cai N, et al. Extracellular Vesicles Secreted by Highly Metastatic Clonal Variants of Osteosarcoma Preferentially Localize to the Lungs and Induce Metastatic Behaviour in Poorly Metastatic Clones. *Oncotarget* (2016) 7(28):43570–87. doi: 10.18632/oncotarget.9781
68. Fan TM, Roberts RD, Lizardo MM. Understanding and Modeling Metastasis Biology to Improve Therapeutic Strategies for Combating Osteosarcoma Progression. *Front Oncol* (2020) 10:13. doi: 10.3389/fonc.2020.00013
69. Ren L, Hong SH, Chen QR, Briggs J, Cassavaugh J, Srinivasan S, et al. Dysregulation of Ezrin Phosphorylation Prevents Metastasis and Alters Cellular Metabolism in Osteosarcoma. *Cancer Res* (2012) 72(4):1001–12. doi: 10.1158/0008-5472.CAN-11-0210
70. Han G, Wang Y, Bi W, Jia J, Wang W, Xu M. Effects of Vascular Endothelial Growth Factor Expression on Pathological Characteristics and Prognosis of Osteosarcoma. *Clin Exp Med* (2016) 16(4):577–84. doi: 10.1007/s10238-015-0382-1
71. Jia SF, Guan H, Duan X, Kleinerman ES. VEGF165 is Necessary to the Metastatic Potential of Fas(-) Osteosarcoma Cells But Will Not Rescue the Fas(+) Cells. *J Exp Ther Oncol* (2008) 7(2):89–97.
72. Wan X, Kim SY, Guenther LM, Mendoza A, Briggs J, Yeung C, et al. Beta4 Integrin Promotes Osteosarcoma Metastasis and Interacts With Ezrin. *Oncogene* (2009) 28(38):3401–11. doi: 10.1038/onc.2009.206
73. Gao YS, Mei J, Tong TL, Hu M, Xue HM, Cai XS. Inhibitory Effects of VEGF-siRNA Mediated by Adenovirus on Osteosarcoma-Bearing Nude Mice. *Cancer Biother Radiopharm* (2009) 24(2):243–7. doi: 10.1089/cbr.2008.0544
74. Ren L, Hong SH, Cassavaugh J, Osborne T, Chou AJ, Kim SY, et al. The Actin-Cytoskeleton Linker Protein Ezrin is Regulated During Osteosarcoma Metastasis by PKC. *Oncogene* (2009) 28(6):792–802. doi: 10.1038/onc.2008.437
75. Folkman J. Role of Angiogenesis in Tumor Growth and Metastasis. *Semin Oncol* (2002) 29(6 Suppl 16):15–8. doi: 10.1053/sonc.2002.37263
76. Lafleur EA, Koshkina NV, Stewart J, Jia SF, Worth LL, Duan X, et al. Increased Fas Expression Reduces the Metastatic Potential of Human Osteosarcoma Cells. *Clin Cancer Res* (2004) 10(23):8114–9. doi: 10.1158/1078-0432.CCR-04-0353
77. Ek ET, Dass CR, Contreras KG, Choong PF. Pigment Epithelium-Derived Factor Overexpression Inhibits Orthotopic Osteosarcoma Growth, Angiogenesis and Metastasis. *Cancer Gene Ther* (2007) 14(7):616–26. doi: 10.1038/sj.cgt.7701044
78. Gordon N, Kleinerman ES. Aerosol Therapy for the Treatment of Osteosarcoma Lung Metastases: Targeting the Fas/FasL Pathway and Rationale for the Use of Gemcitabine. *J Aerosol Med Pulm Drug Deliv* (2010) 23(4):189–96. doi: 10.1089/jamp.2009.0812
79. Ek ET, Dass CR, Contreras KG, Choong PF. Inhibition of Orthotopic Osteosarcoma Growth and Metastasis by Multitargeted Antitumor Activities of Pigment Epithelium-Derived Factor. *Clin Exp Metastasis* (2007) 24(2):93–106. doi: 10.1007/s10585-007-9062-1
80. Dhupkar P, Gordon N, Stewart J, Kleinerman ES. Anti-PD-1 Therapy Redirects Macrophages From an M2 to an M1 Phenotype Inducing Regression of OS Lung Metastases. *Cancer Med* (2018) 7(6):2654–64. doi: 10.1002/cam4.1518
81. Ek ET, Dass CR, Contreras KG, Choong PF. PEDF-Derived Synthetic Peptides Exhibit Antitumor Activity in an Orthotopic Model of Human Osteosarcoma. *J Orthop Res* (2007) 25(12):1671–80. doi: 10.1002/jor.20434
82. Lussier DM, O'Neill L, Nieves LM, McAfee MS, Holechek SA, Collins AW, et al. Enhanced T-Cell Immunity to Osteosarcoma Through Antibody Blockade of PD-1/PD-L1 Interactions. *J Immunother* (2015) 38(3):96–106. doi: 10.1097/CJI.0000000000000065
83. Tang Q, Lu J, Zou C, Shao Y, Chen Y, Narala S, et al. CDH4 is a Novel Determinant of Osteosarcoma Tumorigenesis and Metastasis. *Oncogene* (2018) 37(27):3617–30. doi: 10.1038/s41388-018-0231-2
84. Gvozdenovic A, Arlt MJ, Campanile C, Brennecke P, Husmann K, Li Y, et al. CD44 Enhances Tumor Formation and Lung Metastasis in Experimental Osteosarcoma and is an Additional Predictor for Poor Patient Outcome. *J Bone Miner Res* (2013) 28(4):838–47. doi: 10.1002/jbmr.1817
85. Dass CR, Khachigian LM, Choong PF. C-Jun Is Critical for the Progression of Osteosarcoma: Proof in an Orthotopic Spontaneously Metastasizing Model. *Mol Cancer Res* (2008) 6(8):1289–92. doi: 10.1158/1541-7786.MCR-08-0036
86. Gvozdenovic A, Arlt MJ, Campanile C, Brennecke P, Husmann K, Born W, et al. Silencing of CD44 Gene Expression in Human 143-B Osteosarcoma Cells Promotes Metastasis of Intratibial Tumors in SCID Mice. *PloS One* (2013) 8(4):e60329. doi: 10.1371/journal.pone.0060329
87. Dass CR, Khachigian LM, Choong PF. C-Jun Knockdown Sensitizes Osteosarcoma to Doxorubicin. *Mol Cancer Ther* (2008) 7(7):1909–12. doi: 10.1158/1535-7163.MCT-08-0086
88. Xu JF, Pan XH, Zhang SJ, Zhao C, Qiu BS, Gu HF, et al. CD47 Blockade Inhibits Tumor Progression Human Osteosarcoma in Xenograft Models. *Oncotarget* (2015) 6(27):23662–70. doi: 10.18632/oncotarget.4282
89. Dass CR, Friedhuber AM, Khachigian LM, Dunstan DE, Choong PF. Downregulation of C-Jun Results in Apoptosis-Mediated Anti-Osteosarcoma Activity in an Orthotopic Model. *Cancer Biol Ther* (2008) 7(7):1033–6. doi: 10.4161/cbt.7.7.6037
90. Manara MC, Bernard G, Lollini PL, Nanni P, Zuntini M, Landuzzi L, et al. CD99 Acts as an Oncosuppressor in Osteosarcoma. *Mol Biol Cell* (2006) 17(4):1910–21. doi: 10.1091/mbc.e05-10-0971
91. Sabile AA, Arlt MJ, Muff R, Bode B, Langsam B, Bertz J, et al. Cyr61 Expression in Osteosarcoma Indicates Poor Prognosis and Promotes Intratibial Growth and Lung Metastasis in Mice. *J Bone Miner Res* (2012) 27(1):58–67. doi: 10.1002/jbmr.535
92. Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR, et al. CD117 and Stro-1 Identify Osteosarcoma Tumor-Initiating Cells Associated With Metastasis and Drug Resistance. *Cancer Res* (2010) 70(11):4602–12. doi: 10.1158/0008-5472.CAN-09-3463
93. Habel N, Vilalta M, Bawa O, Opolon P, Blanco J, Fromigue O. Cyr61 Silencing Reduces Vascularization and Dissemination of Osteosarcoma Tumors. *Oncogene* (2015) 34(24):3207–13. doi: 10.1038/onc.2014.232
94. He A, Yang X, Huang Y, Feng T, Wang Y, Sun Y, et al. CD133(+) CD44(+) Cells Mediate in the Lung Metastasis of Osteosarcoma. *J Cell Biochem* (2015) 116(8):1719–29. doi: 10.1002/jcb.25131
95. Habel N, Stefanovska B, Carene D, Patino-Garcia A, Lecanda F, Fromigue O. CYR61 Triggers Osteosarcoma Metastatic Spreading *via* an IGF1Rbeta-Dependent EMT-Like Process. *BMC Cancer* (2019) 19(1):62. doi: 10.1186/s12885-019-5282-4
96. Zhang Y, Ma Q, Liu T, Guan G, Zhang K, Chen J, et al. Interleukin-6 Suppression Reduces Tumour Self-Seeding by Circulating Tumour Cells in a Human Osteosarcoma Nude Mouse Model. *Oncotarget* (2016) 7(1):446–58. doi: 10.18632/oncotarget.6371
97. Tu B, Du L, Fan QM, Tang Z, Tang TT. STAT3 Activation by IL-6 From Mesenchymal Stem Cells Promotes the Proliferation and Metastasis of Osteosarcoma. *Cancer Lett* (2012) 325(1):80–8. doi: 10.1016/j.canlet.2012.06.006
98. Kularatne SA, Deshmukh V, Ma J, Tardif V, Lim RK, Pugh HM, et al. A CXCR4-Targeted Site-Specific Antibody-Drug Conjugate. *Angew Chem Int Ed Engl* (2014) 53(44):11863–7. doi: 10.1002/anie.201408103
99. Zhang Z, Wang F, Li Q, Zhang H, Cui Y, Ma C, et al. CD151 Knockdown Inhibits Osteosarcoma Metastasis Through the GSK-3beta/Beta-Catenin/MMP9 Pathway. *Oncol Rep* (2016) 35(3):1764–70. doi: 10.3892/or.2015.4517
100. Neklyudova O, Arlt MJ, Brennecke P, Thelen M, Gvozdenovic A, Kuzmanov A, et al. Altered CXCL12 Expression Reveals a Dual Role of CXCR4 in Osteosarcoma Primary Tumor Growth and Metastasis. *J Cancer Res Clin Oncol* (2016) 142(8):1739–50. doi: 10.1007/s00432-016-2185-5
101. Itoh H, Kadomatsu T, Tanoue H, Yugami M, Miyata K, Endo M, et al. TET2-Dependent IL-6 Induction Mediated by the Tumor Microenvironment Promotes Tumor Metastasis in Osteosarcoma. *Oncogene* (2018) 37(22):2903–20. doi: 10.1038/s41388-018-0160-0
102. Dang H, Wu W, Wang B, Cui C, Niu J, Chen J, et al. CXCL5 Plays a Promoting Role in Osteosarcoma Cell Migration and Invasion in Autocrine- and Paracrine-Dependent Manners. *Oncol Res* (2017) 25(2):177–86. doi: 10.3727/096504016X14732772150343

103. Wang M, Wang L, Ren T, Xu L, Wen Z. IL-17a/IL-17RA Interaction Promoted Metastasis of Osteosarcoma Cells. *Cancer Biol Ther* (2013) 14 (2):155–63. doi: 10.4161/cbt.22955
104. Du L, Han XG, Tu B, Wang MQ, Qiao H, Zhang SH, et al. CXCR1/Akt Signaling Activation Induced by Mesenchymal Stem Cell-Derived IL-8 Promotes Osteosarcoma Cell Anoikis Resistance and Pulmonary Metastasis. *Cell Death Dis* (2018) 9(7):714. doi: 10.1038/s41419-018-0745-0
105. Segaliny AI, Mohamadi A, Dizier B, Lokajczyk A, Brion R, Lanel R, et al. Interleukin-34 Promotes Tumor Progression and Metastatic Process in Osteosarcoma Through Induction of Angiogenesis and Macrophage Recruitment. *Int J Cancer* (2015) 137(1):73–85. doi: 10.1002/ijc.29376
106. Brennecke P, Arlt MJ, Campanile C, Husmann K, Gvozdenovic A, Apuzzo T, et al. CXCR4 Antibody Treatment Suppresses Metastatic Spread to the Lung of Intratibial Human Osteosarcoma Xenografts in Mice. *Clin Exp Metastasis* (2014) 31(3):339–49. doi: 10.1007/s10585-013-9632-3
107. Akiyama T, Choong PF, Dass CR. RANK-Fc Inhibits Malignancy *via* Inhibiting ERK Activation and Evoking Caspase-3-Mediated Anoikis in Human Osteosarcoma Cells. *Clin Exp Metastasis* (2010) 27(4):207–15. doi: 10.1007/s10585-010-9319-y
108. Zhang P, Dong L, Yan K, Long H, Yang TT, Dong MQ, et al. CXCR4-Mediated Osteosarcoma Growth and Pulmonary Metastasis is Promoted by Mesenchymal Stem Cells Through VEGF. *Oncol Rep* (2013) 30(4):1753–61. doi: 10.3892/or.2013.2619
109. Akiyama T, Dass CR, Shinoda Y, Kawano H, Tanaka S, Choong PF. Systemic RANK-Fc Protein Therapy is Efficacious Against Primary Osteosarcoma Growth in a Murine Model *via* Activity Against Osteoclasts. *J Pharm Pharmacol* (2010) 62(4):470–6. doi: 10.1211/jpp.62.04.0009
110. Goguet-Surmenian E, Richard-Fiardo P, Guillemot E, Benchetrit M, Gomez-Brouchet A, Buzzo P, et al. CXCR7-Mediated Progression of Osteosarcoma in the Lungs. *Br J Cancer* (2013) 109(6):1579–85. doi: 10.1038/bjc.2013.482
111. Lamoureux F, Picarda G, Rousseau J, Gourden C, Battaglia S, Charrier C, et al. Therapeutic Efficacy of Soluble Receptor Activator of Nuclear Factor-Kappa B-Fc Delivered by Nonviral Gene Transfer in a Mouse Model of Osteolytic Osteosarcoma. *Mol Cancer Ther* (2008) 7(10):3389–98. doi: 10.1158/1535-7163.MCT-08-0497
112. de Nigris F, Rossiello R, Schiano C, Arra C, Williams-Ignarro S, Barbieri A, et al. Deletion of Yin Yang 1 Protein in Osteosarcoma Cells on Cell Invasion and CXCR4/angiogenesis and Metastasis. *Cancer Res* (2008) 68(6):1797–808. doi: 10.1158/0008-5472.CAN-07-5582
113. Picarda G, Lamoureux F, Geffroy L, Delepine P, Montier T, Laud K, et al. Preclinical Evidence That Use of TRAIL in Ewing's Sarcoma and Osteosarcoma Therapy Inhibits Tumor Growth, Prevents Osteolysis, and Increases Animal Survival. *Clin Cancer Res* (2010) 16(8):2363–74. doi: 10.1158/1078-0432.CCR-09-1779
114. Gozo MC, Jia D, Aspuria PJ, Cheon DJ, Miura N, Walts AE, et al. FOXC2 Augments Tumor Propagation and Metastasis in Osteosarcoma. *Oncotarget* (2016) 7(42):68792–802. doi: 10.18632/oncotarget.11990
115. Cao Y, Zhou Z, de Crombrugghe B, Nakashima K, Guan H, Duan X, et al. Osterix, a Transcription Factor for Osteoblast Differentiation, Mediates Antitumor Activity in Murine Osteosarcoma. *Cancer Res* (2005) 65 (4):1124–8. doi: 10.1158/0008-5472.CAN-04-2128
116. Brennecke P, Arlt MJ, Muff R, Campanile C, Gvozdenovic A, Husmann K, et al. Expression of the Chemokine Receptor CXCR7 in CXCR4-Expressing Human 143B Osteosarcoma Cells Enhances Lung Metastasis of Intratibial Xenografts in SCID Mice. *PloS One* (2013) 8(9):e74045. doi: 10.1371/journal.pone.0074045
117. Lamora A, Talbot J, Bougras G, Amiaud J, Leduc M, Chesneau J, et al. Overexpression of Smad7 Blocks Primary Tumor Growth and Lung Metastasis Development in Osteosarcoma. *Clin Cancer Res* (2014) 20 (19):5097–112. doi: 10.1158/1078-0432.CCR-13-3191
118. Perissinotto E, Cavalloni G, Leone F, Fonsato V, Mitola S, Grignani G, et al. Involvement of Chemokine Receptor 4/Stromal Cell-Derived Factor 1 System During Osteosarcoma Tumor Progression. *Clin Cancer Res* (2005) 11(2 Pt 1):490–7.
119. Endo-Munoz L, Cumming A, Rickwood D, Wilson D, Cueva C, Ng C, et al. Loss of Osteoclasts Contributes to Development of Osteosarcoma Pulmonary Metastases. *Cancer Res* (2010) 70(18):7063–72. doi: 10.1158/0008-5472.CAN-09-4291
120. Rodriguez Calleja L, Jacques C, Lamoureux F, Baud'huin M, Tellez Gabriel M, Quillard T, et al. DeltaNp63alpha Silences a miRNA Program to Aberrantly Initiate a Wound-Healing Program That Promotes TGFbeta-Induced Metastasis. *Cancer Res* (2016) 76(11):3236–51. doi: 10.1158/0008-5472.CAN-15-2317
121. Gross AC, Cam H, Phelps DA, Saraf AJ, Bid HK, Cam M, et al. IL-6 and CXCL8 Mediate Osteosarcoma-Lung Interactions Critical to Metastasis. *JCI Insight* 3(16):e99791. doi: 10.1172/jci.insight.99791
122. Gvozdenovic A, Boro A, Meier D, Bode-Lesniewska B, Born W, Muff R, et al. Targeting Alphavbeta3 and Alphavbeta5 Integrins Inhibits Pulmonary Metastasis in an Intratibial Xenograft Osteosarcoma Mouse Model. *Oncotarget* (2016) 7(34):55141–54. doi: 10.18632/oncotarget.10461
123. Bid HK, Roberts RD, Cam M, Audino A, Kurmasheva RT, Lin J, et al. DeltaNp63 Promotes Pediatric Neuroblastoma and Osteosarcoma by Regulating Tumor Angiogenesis. *Cancer Res* (2014) 74(1):320–9. doi: 10.1158/0008-5472.CAN-13-0894
124. Li R, Shi Y, Zhao S, Shi T, Zhang G. NF-kappaB Signaling and Integrin-Beta1 Inhibition Attenuates Osteosarcoma Metastasis *via* Increased Cell Apoptosis. *Int J Biol Macromol* (2019) 123:1035–43. doi: 10.1016/j.ijbiomac.2018.11.003
125. Li Y, Liao Q, Li K, Zhong D, Weng X, Mi M. Knockdown of Endothelin A Receptor Expression Inhibits Osteosarcoma Pulmonary Metastasis in an Orthotopic Xenograft Mouse Model. *Mol Med Rep* (2012) 5(6):1391–5. doi: 10.3892/mmr.2012.842
126. Pourebrahim R, Zhang Y, Liu B, Gao R, Xiong S, Lin PP, et al. Integrative Genome Analysis of Somatic P53 Mutant Osteosarcomas Identifies Ets2-Dependent Regulation of Small Nucleolar RNAs by Mutant P53 Protein. *Genes Dev* (2017) 31(18):1847–57. doi: 10.1101/gad.304972.117
127. Zhang D, Jiang F, Wang X, Li G. Knockdown of SALL4 Inhibits Proliferation, Migration, and Invasion in Osteosarcoma Cells. *Oncol Res* (2017) 25(5):763–71. doi: 10.3727/096504016X14772402056137
128. Zhang Y, Hu Q, Li G, Li L, Liang S, Zhang Y, et al. ONZIN Upregulation by Mutant P53 Contributes to Osteosarcoma Metastasis Through the CXCL5-MAPK Signaling Pathway. *Cell Physiol Biochem* (2018) 48(3):1099–111. doi: 10.1159/000491976
129. Yong BC, Lu JC, Xie XB, Su Q, Tan PX, Tang QL, et al. LDOC1 Regulates Wnt5a Expression and Osteosarcoma Cell Metastasis and is Correlated With the Survival of Osteosarcoma Patients. *Tumour Biol* (2017) 39 (2):1010428317691188. doi: 10.1177/1010428317691188
130. Luther GA, Lamplot J, Chen X, Rames R, Wagner ER, Liu X, et al. IGFBP5 Domains Exert Distinct Inhibitory Effects on the Tumorigenicity and Metastasis of Human Osteosarcoma. *Cancer Lett* (2013) 336(1):222–30. doi: 10.1016/j.canlet.2013.05.002
131. Wang J, Ni J, Song D, Ding M, Huang J, Li W, et al. MAT1 Facilitates the Lung Metastasis of Osteosarcoma Through Upregulation of AKT1 Expression. *Life Sci* (2019) 234:116771. doi: 10.1016/j.lfs.2019.116771
132. Su Y, Wagner ER, Luo Q, Huang J, Chen L, He BC, et al. Insulin-Like Growth Factor Binding Protein 5 Suppresses Tumor Growth and Metastasis of Human Osteosarcoma. *Oncogene* (2011) 30(37):3907–17. doi: 10.1038/onc.2011.97
133. Zeng H, Zhang JM, Du Y, Wang J, Ren Y, Li M, et al. Crosstalk Between ATF4 and MTA1/HDAC1 Promotes Osteosarcoma Progression. *Oncotarget* (2016) 7(6):7329–42. doi: 10.18632/oncotarget.6940
134. Wang S, Zhang D, Han S, Gao P, Liu C, Li J, et al. Fibulin-3 Promotes Osteosarcoma Invasion and Metastasis by Inducing Epithelial to Mesenchymal Transition and Activating the Wnt/beta-Catenin Signaling Pathway. *Sci Rep* (2017) 7(1):6215. doi: 10.1038/s41598-017-06353-2
135. Lu J, Song G, Tang Q, Zou C, Han F, Zhao Z, et al. IRX1 Hypomethylation Promotes Osteosarcoma Metastasis *via* Induction of CXCL14/NF-kappaB Signaling. *J Clin Invest* (2015) 125(5):1839–56. doi: 10.1172/JCI78437
136. Zhang D, Wang S, Chen J, Liu H, Lu J, Jiang H, et al. Fibulin-4 Promotes Osteosarcoma Invasion and Metastasis by Inducing Epithelial to Mesenchymal Transition *via* the PI3K/Akt/mTOR Pathway. *Int J Oncol* (2017) 50(5):1513–30. doi: 10.3892/ijo.2017.3921
137. Manara MC, Baldini N, Serra M, Lollini PL, De Giovanni C, Vaccari M, et al. Reversal of Malignant Phenotype in Human Osteosarcoma Cells Transduced With the Alkaline Phosphatase Gene. *Bone* (2000) 26(3):215–20. doi: 10.1016/s8756-3282(99)00266-5

138. Zhang G, Li M, Jin J, Bai Y, Yang C. Knockdown of S100A4 Decreases Tumorigenesis and Metastasis in Osteosarcoma Cells by Repression of Matrix Metalloproteinase-9. *Asian Pac J Cancer Prev* (2011) 12(8):2075–80.
139. Li YJ, Dong BK, Fan M, Jiang WX. BTG2 Inhibits the Proliferation and Metastasis of Osteosarcoma Cells by Suppressing the PI3K/AKT Pathway. *Int J Clin Exp Pathol* (2015) 8(10):12410–8.
140. Fujiwara M, Kashima TG, Kunita A, Kii I, Komura D, Grigoriadis AE, et al. Stable Knockdown of S100A4 Suppresses Cell Migration and Metastasis of Osteosarcoma. *Tumour Biol* (2011) 32(3):611–22. doi: 10.1007/s13277-011-0160-y
141. Chen MW, Wu XJ. SLC25A22 Promotes Proliferation and Metastasis of Osteosarcoma Cells *via* the PTEN Signaling Pathway. *Technol Cancer Res Treat* (2018) 17:1533033818811143. doi: 10.1177/1533033818811143
142. Qin Y, Ye J, Zhao F, Hu S, Wang S. TRIM2 Regulates the Development and Metastasis of Tumorous Cells of Osteosarcoma. *Int J Oncol* (2018) 53 (4):1643–56. doi: 10.3892/ijo.2018.4494
143. Chien MH, Lee WJ, Yang YC, Tan P, Pan KF, Liu YC, et al. N-Alpha-Acetyltransferase 10 Protein Promotes Metastasis by Stabilizing Matrix Metalloproteinase-2 Protein in Human Osteosarcomas. *Cancer Lett* (2018) 433:86–98. doi: 10.1016/j.canlet.2018.06.033
144. Chen Y, Guo Y, Yang H, Shi G, Xu G, Shi J, et al. TRIM66 Overexpresssion Contributes to Osteosarcoma Carcinogenesis and Indicates Poor Survival Outcome. *Oncotarget* (2015) 6(27):23708–19. doi: 10.18632/oncotarget.4291
145. Li Y, Nakka M, Kelly AJ, Lau CC, Krailo M, Barkauskas DA, et al. P27 Is a Candidate Prognostic Biomarker and Metastatic Promoter in Osteosarcoma. *Cancer Res* (2016) 76(13):4002–11. doi: 10.1158/0008-5472.CAN-15-3189
146. Cao J, Wang Y, Dong R, Lin G, Zhang N, Wang J, et al. Hypoxia-Induced WSB1 Promotes the Metastatic Potential of Osteosarcoma Cells. *Cancer Res* (2015) 75(22):4839–51. doi: 10.1158/0008-5472.CAN-15-0711
147. Endo-Munoz L, Cai N, Cumming A, Macklin R, Merida de Long L, Topkas E, et al. Progression of Osteosarcoma From a Non-Metastatic to a Metastatic Phenotype Is Causally Associated With Activation of an Autocrine and Paracrine uPA Axis. *PloS One* (2015) 10(8):e0133592. doi: 10.1371/journal.pone.0133592
148. Fukaya Y, Ishiguro N, Senga T, Ichigotani Y, Sohara Y, Tsutsui M, et al. A Role for PI3K-Akt Signaling in Pulmonary Metastatic Nodule Formation of the Osteosarcoma Cell Line, LM8. *Oncol Rep* (2005) 14(4):847–52. doi: 10.3892/or.14.4.847
149. Shi QM, Luo J, Wu K, Yin M, Gu YR, Cheng XG. High Level of alphaB-Crystallin Contributes to the Progression of Osteosarcoma. *Oncotarget* (2016) 7(8):9007–16. doi: 10.18632/oncotarget.6928
150. Liu JF, Tsao YT, Hou CH. Amphiregulin Enhances Intercellular Adhesion Molecule-1 Expression and Promotes Tumor Metastasis in Human Osteosarcoma. *Oncotarget* (2015) 6(38):40880–95. doi: 10.18632/oncotarget.5679
151. Lv YF, Yan GN, Meng G, Zhang X, Guo QN. Enhancer of Zeste Homolog 2 Silencing Inhibits Tumor Growth and Lung Metastasis in Osteosarcoma. *Sci Rep* (2015) 5:12999. doi: 10.1038/srep12999
152. Guo YS, Zhao R, Ma J, Cui W, Sun Z, Gao B, et al. Betaig-H3 Promotes Human Osteosarcoma Cells Metastasis by Interacting With Integrin Alpha2beta1 and Activating PI3K Signaling Pathway. *PloS One* (2014) 9 (3):e90220. doi: 10.1371/journal.pone.0090220
153. Baglio SR, Lagerweij T, Perez-Lanzon M, Ho XD, Leveille N, Melo SA, et al. Blocking Tumor-Educated MSC Paracrine Activity Halts Osteosarcoma Progression. *Clin Cancer Res* (2017) 23(14):3721–33. doi: 10.1158/1078-0432.CCR-16-2726
154. Ren K, Zhang J, Gu X, Wu S, Shi X, Ni Y, et al. Migration-Inducing Gene-7 Independently Predicts Poor Prognosis of Human Osteosarcoma and Is Associated With Vasculogenic Mimicry. *Exp Cell Res* (2018) 369(1):80–9. doi: 10.1016/j.yexcr.2018.05.008
155. Liu J, Luo B, Zhao M. Bmi1targeting Suppresses Osteosarcoma Aggressiveness Through the NFkappaB Signaling Pathway. *Mol Med Rep* (2017) 16(6):7949–58. doi: 10.3892/mmr.2017.7660
156. Han W, Liu J. Epigenetic Silencing of the Wnt Antagonist APCDD1 by Promoter DNA Hyper-Methylation Contributes to Osteosarcoma Cell Invasion and Metastasis. *Biochem Biophys Res Commun* (2017) 491(1):91–7. doi: 10.1016/j.bbrc.2017.07.049
157. El-Naggar AM, Clarkson PW, Negri GL, Turgu B, Zhang F, Anglesio MS, et al. HACE1 is a Potential Tumor Suppressor in Osteosarcoma. *Cell Death Dis* (2019) 10(1):21. doi: 10.1038/s41419-018-1276-4
158. Yue B, Ren QX, Su T, Wang LN, Zhang L. ERK5 Silencing Inhibits Invasion of Human Osteosarcoma Cell via Modulating the Slug/MMP-9 Pathway. *Eur Rev Med Pharmacol Sci* (2014) 18(18):2640–7.
159. Sun W, Wang W, Lei J, Li H, Wu Y. Actin-Like Protein 6A is a Novel Prognostic Indicator Promoting Invasion and Metastasis in Osteosarcoma. *Oncol Rep* (2017) 37(4):2405–17. doi: 10.3892/or.2017.5473
160. Contaldo C, Myers TJ, Zucchini C, Manara MC, Chiodoni C, Colombo MP, et al. Expression Levels of Insulin Receptor Substrate-1 Modulate the Osteoblastic Differentiation of Mesenchymal Stem Cells and Osteosarcoma Cells. *Growth Factors* (2014) 32(1):41–52. doi: 10.3109/08977194.2013.870168
161. Zhao Z, Wu MS, Zou C, Tang Q, Lu J, Liu D, et al. Downregulation of MCT1 Inhibits Tumor Growth, Metastasis and Enhances Chemotherapeutic Efficacy in Osteosarcoma Through Regulation of the NF-kappaB Pathway. *Cancer Lett* (2014) 342(1):150–8. doi: 10.1016/j.canlet.2013.08.042
162. Levings PP, McGarry SV, Currie TP, Nickerson DM, McClellan S, Ghivizzani SC, et al. Expression of an Exogenous Human Oct-4 Promoter Identifies Tumor-Initiating Cells in Osteosarcoma. *Cancer Res* (2009) 69 (14):5648–55. doi: 10.1158/0008-5472.CAN-08-3580
163. Wang Z, Cao CJ, Huang LL, Ke ZF, Luo CJ, Lin ZW, et al. EFEMP1 Promotes the Migration and Invasion of Osteosarcoma *via* MMP-2 With Induction by AEG-1 *via* NF-kappaB Signaling Pathway. *Oncotarget* (2015) 6(16):14191–208. doi: 10.18632/oncotarget.3691
164. Arlt MJ, Kuzmanov A, Snedeker JG, Fuchs B, Silvan U, Sabile AA. Fascin-1 Enhances Experimental Osteosarcoma Tumor Formation and Metastasis and is Related to Poor Patient Outcome. *BMC Cancer* (2019) 19(1):83. doi: 10.1186/s12885-019-5303-3
165. Zandueta C, Ormazabal C, Perurena N, Martinez-Canarias S, Zalacain M, Julian MS, et al. Matrix-Gla Protein Promotes Osteosarcoma Lung Metastasis and Associates With Poor Prognosis. *J Pathol* (2016) 239 (4):438–49. doi: 10.1002/path.4740
166. Long F, Cai X, Luo W, Chen L, Li K. Role of Aldolase A in Osteosarcoma Progression and Metastasis: *In Vitro* and *In Vivo* Evidence. *Oncol Rep* (2014) 32(5):2031–7. doi: 10.3892/or.2014.3473
167. Xu L, Xia C, Sheng F, Sun Q, Xiong J, Wang S. CEP55 Promotes the Proliferation and Invasion of Tumour Cells *via* the AKT Signalling Pathway in Osteosarcoma. *Carcinogenesis* (2018) 39(4):623–31. doi: 10.1093/carcin/bgy017
168. Ma C, Zhang Z, Cui Y, Yuan H, Wang F. Silencing FAT10 Inhibits Metastasis of Osteosarcoma. *Int J Oncol* (2016) 49(2):666–74. doi: 10.3892/ijo.2016.3549
169. Ren L, Mendoza A, Zhu J, Briggs JW, Halsey C, Hong ES, et al. Characterization of the Metastatic Phenotype of a Panel of Established Osteosarcoma Cells. *Oncotarget* (2015) 6(30):29469–81. doi: 10.18632/oncotarget.5177
170. Niinaka Y, Harada K, Fujimuro M, Oda M, Haga A, Hosoki M, et al. Silencing of Autocrine Motility Factor Induces Mesenchymal-to-Epithelial Transition and Suppression of Osteosarcoma Pulmonary Metastasis. *Cancer Res* (2010) 70(22):9483–93. doi: 10.1158/0008-5472.CAN-09-3880
171. Yu W, Zhang Z, Min D, Yang Q, Du X, Tang L, et al. Mediator of RNA Polymerase II Transcription Subunit 19 Promotes Osteosarcoma Growth and Metastasis and Associates With Prognosis. *Eur J Cancer* (2014) 50 (6):1125–36. doi: 10.1016/j.ejca.2014.01.030
172. Zhang Y, Cheng H, Li W, Wu H, Yang Y. Highly-Expressed P2X7 Receptor Promotes Growth and Metastasis of Human HOS/MNNG Osteosarcoma Cells *via* PI3K/Akt/GSK3beta/beta-Catenin and mTOR/HIF1alpha/VEGF Signaling. *Int J Cancer* (2019) 145(4):1068–82. doi: 10.1002/ijc.32207
173. Hou CH, Yang RS, Tsao YT. Connective Tissue Growth Factor Stimulates Osteosarcoma Cell Migration and Induces Osteosarcoma Metastasis by Upregulating VCAM-1 Expression. *Biochem Pharmacol* (2018) 155:71–81. doi: 10.1016/j.bcp.2018.06.015
174. Zhao SJ, Jiang YQ, Xu NW, Li Q, Zhang Q, Wang SY, et al. SPARCL1 Suppresses Osteosarcoma Metastasis and Recruits Macrophages by Activation of Canonical WNT/beta-Catenin Signaling Through Stabilization of the WNT-Receptor Complex. *Oncogene* (2018) 37(8):1049–61. doi: 10.1038/onc.2017.403

175. McManus M, Kleinerman E, Yang Y, Livingston JA, Mortus J, Rivera R, et al. Hes4: A Potential Prognostic Biomarker for Newly Diagnosed Patients With High-Grade Osteosarcoma. *Pediatr Blood Cancer* (2017) 64(5):10. doi: 10.1002/pbc.26318
176. Dai H, Lv YF, Yan GN, Meng G, Zhang X, Guo QN. RanBP9/TSSC3 Complex Cooperates to Suppress Anoikis Resistance and Metastasis *via* Inhibiting Src-Mediated Akt Signaling in Osteosarcoma. *Cell Death Dis* (2016) 7(12):e2572. doi: 10.1038/cddis.2016.436
177. Zhang P, Yang Y, Zweidler-McKay PA, Hughes DP. Critical Role of Notch Signaling in Osteosarcoma Invasion and Metastasis. *Clin Cancer Res* (2008) 14(10):2962–9. doi: 10.1158/1078-0432.CCR-07-1992
178. Brun J, Dieudonne FX, Marty C, Muller J, Schule R, Patino-Garcia A, et al. FHL2 Silencing Reduces Wnt Signaling and Osteosarcoma Tumorigenesis *In Vitro* and In Vivo. *PloS One* (2013) 8(1):e55034. doi: 10.1371/journal.pone.0055034
179. Hughes DP. How the NOTCH Pathway Contributes to the Ability of Osteosarcoma Cells to Metastasize. *Cancer Treat Res* (2009) 152:479–96. doi: 10.1007/978-1-4419-0284-9_28
180. Cheng Q, Yin G. Cullin-1 Regulates MG63 Cell Proliferation and Metastasis and Is a Novel Prognostic Marker of Osteosarcoma. *Int J Biol Markers* (2017) 32(2):e202–9. doi: 10.5301/jbm.5000247
181. Zhang Y, Zvi YS, Batko B, Zaphiros N, O'Donnell EF, Wang J, et al. Down-Regulation of Skp2 Expression Inhibits Invasion and Lung Metastasis in Osteosarcoma. *Sci Rep* (2018) 8(1):14294. doi: 10.1038/s41598-018-32428-9
182. Morrow JJ, Mendoza A, Koyen A, Lizardo MM, Ren L, Waybright TJ, et al. mTOR Inhibition Mitigates Enhanced mRNA Translation Associated With the Metastatic Phenotype of Osteosarcoma Cells In Vivo. *Clin Cancer Res* (2016) 22(24):6129–41. doi: 10.1158/1078-0432.CCR-16-0326
183. Zhang N, Xie T, Xian M, Wang YJ, Li HY, Ying MD, et al. SIRT1 Promotes Metastasis of Human Osteosarcoma Cells. *Oncotarget* (2016) 7(48):79654–69. doi: 10.18632/oncotarget.12916
184. Zhang F, Yan T, Guo W, Sun K, Wang S, Bao X, et al. Novel Oncogene COPS3 Interacts With Beclin1 and Raf-1 to Regulate Metastasis of Osteosarcoma Through Autophagy. *J Exp Clin Cancer Res* (2018) 37 (1):135. doi: 10.1186/s13046-018-0791-6
185. Lv YF, Dai H, Yan GN, Meng G, Zhang X, Guo QN. Downregulation of Tumor Suppressing STF cDNA 3 Promotes Epithelial-Mesenchymal Transition and Tumor Metastasis of Osteosarcoma by the Wnt/GSK-3beta/Beta-Catenin/Snail Signaling Pathway. *Cancer Lett* (2016) 373 (2):164–73. doi: 10.1016/j.canlet.2016.01.046
186. Techavichit P, Gao Y, Kurenbekova L, Shuck R, Donehower LA, Yustein JT. Secreted Frizzled-Related Protein 2 (Sfrp2) Promotes Osteosarcoma Invasion and Metastatic Potential. *BMC Cancer* (2016) 16(1):869. doi: 10.1186/s12885-016-2909-6
187. Zhao GS, Gao ZR, Zhang Q, Tang XF, Lv YF, Zhang ZS, et al. TSSC3 Promotes Autophagy *via* Inactivating the Src-Mediated PI3K/Akt/mTOR Pathway to Suppress Tumorigenesis and Metastasis in Osteosarcoma, and Predicts a Favorable Prognosis. *J Exp Clin Cancer Res* (2018) 37(1):188. doi: 10.1186/s13046-018-0856-6
188. Ma J, Gao W, Gao J. sCLU as Prognostic Biomarker and Therapeutic Target in Osteosarcoma. *Bioengineered* (2019) 10(1):229–39. doi: 10.1080/21655979.2019.1621136
189. Jin PY, Lu HJ, Tang Y, Fan SH, Zhang ZF, Wang Y, et al. The Effect of DNA-PKcs Gene Silencing on Proliferation, Migration, Invasion and Apoptosis, and *In Vivo* Tumorigenicity of Human Osteosarcoma MG-63 Cells. *BioMed Pharmacother* (2017) 96:1324–34. doi: 10.1016/j.biopha.2017.11.079
190. Hou CH, Lin FL, Tong KB, Hou SM, Liu JF. Transforming Growth Factor Alpha Promotes Osteosarcoma Metastasis by ICAM-1 and PI3K/Akt Signaling Pathway. *Biochem Pharmacol* (2014) 89(4):453–63. doi: 10.1016/j.bcp.2014.03.010
191. Tieken C, Verboom MC, Ruf W, Gelderblom H, Bovee JV, Reitsma PH, et al. Tissue Factor Associates With Survival and Regulates Tumour Progression in Osteosarcoma. *Thromb Haemost* (2016) 115(5):1025–33. doi: 10.1160/TH15-07-0541
192. Chang H, Dong T, Ma X, Zhang T, Chen Z, Yang Z, et al. Spondin 1 Promotes Metastatic Progression Through Fak and Src Dependent Pathway in Human Osteosarcoma. *Biochem Biophys Res Commun* (2015) 464(1):45–50. doi: 10.1016/j.bbrc.2015.05.092
193. Yu L, Liu S, Guo W, Zhang B, Liang Y, Feng Q. Upregulation of Mad2 Facilitates *In Vivo* and *In Vitro* Osteosarcoma Progression. *Oncol Rep* (2012) 28(6):2170–6. doi: 10.3892/or.2012.2032
194. El-Naggar AM, Veinotte CJ, Cheng H, Grunewald TG, Negri GL, Somasekharan SP, et al. Translational Activation of HIF1alpha by YB-1 Promotes Sarcoma Metastasis. *Cancer Cell* (2015) 27(5):682–97. doi: 10.1016/j.ccell.2015.04.003
195. Rubin EM, Guo Y, Tu K, Xie J, Zi X, Hoang BH. Wnt Inhibitory Factor 1 Decreases Tumorigenesis and Metastasis in Osteosarcoma. *Mol Cancer Ther* (2010) 9(3):731–41. doi: 10.1158/1535-7163.MCT-09-0147
196. Hu X, Liu Y, Qin C, Pan Z, Luo J, Yu A, et al. Up-Regulated Isocitrate Dehydrogenase 1 Suppresses Proliferation, Migration and Invasion in Osteosarcoma: *In Vitro* and In Vivo. *Cancer Lett* (2014) 346(1):114–21. doi: 10.1016/j.canlet.2013.12.020
197. Cantiani L, Manara MC, Zucchini C, De Sanctis P, Zuntini M, Valvassori L, et al. Caveolin-1 Reduces Osteosarcoma Metastases by Inhibiting C-Src Activity and Met Signaling. *Cancer Res* (2007) 67(16):7675–85. doi: 10.1158/0008-5472.CAN-06-4697
198. Severe N, Dieudonne FX, Marty C, Modrowski D, Patino-Garcia A, Lecanda F, et al. Targeting the E3 Ubiquitin Casitas B-Lineage Lymphoma Decreases Osteosarcoma Cell Growth and Survival and Reduces Tumorigenesis. *J Bone Miner Res* (2012) 27(10):2108–17. doi: 10.1002/jbmr.1667
199. Zhang W, Zhao JM, Lin J, Hu CZ, Zhang WB, Yang WL, et al. Adaptive Fibrogenic Reprogramming of Osteosarcoma Stem Cells Promotes Metastatic Growth. *Cell Rep* (2018) 24(5):1266–1277 e5. doi: 10.1016/j.celrep.2018.06.103
200. Koshkina NV, Khanna C, Mendoza A, Guan H, DeLauter L, Kleinerman ES. Fas-Negative Osteosarcoma Tumor Cells Are Selected During Metastasis to the Lungs: The Role of the Fas Pathway in the Metastatic Process of Osteosarcoma. *Mol Cancer Res* (2007) 5(10):991–9. doi: 10.1158/1541-7786.MCR-07-0007
201. Gordon N, Kleinerman ES. The Role of Fas/FasL in the Metastatic Potential of Osteosarcoma and Targeting This Pathway for the Treatment of Osteosarcoma Lung Metastases. *Cancer Treat Res* (2009) 152:497–508. doi: 10.1007/978-1-4419-0284-9_29
202. Huang G, Nishimoto K, Yang Y, Kleinerman ES. Participation of the Fas/FasL Signaling Pathway and the Lung Microenvironment in the Development of Osteosarcoma Lung Metastases. *Adv Exp Med Biol* (2014) 804:203–17. doi: 10.1007/978-3-319-04843-7_11
203. Gordon N, Koshkina NV, Jia SF, Khanna C, Mendoza A, Worth LL, et al. Corruption of the Fas Pathway Delays the Pulmonary Clearance of Murine Osteosarcoma Cells, Enhances Their Metastatic Potential, and Reduces the Effect of Aerosol Gemcitabine. *Clin Cancer Res* (2007) 13(15 Pt 1):4503–10. doi: 10.1158/1078-0432.CCR-07-0313
204. Koshkina N, Yang Y, Kleinerman ES. The Fas/FasL Signaling Pathway: Its Role in the Metastatic Process and as a Target for Treating Osteosarcoma Lung Metastases. *Adv Exp Med Biol* (2020) 1258:177–87. doi: 10.1007/978-3-030-43085-6_12
205. Rodriguez COJr., Crabbs TA, Wilson DW, Cannan VA, Skorupski KA, Gordon N, et al. Aerosol Gemcitabine: Preclinical Safety and *In Vivo* Antitumor Activity in Osteosarcoma-Bearing Dogs. *J Aerosol Med Pulm Drug Deliv* (2010) 23(4):197–206. doi: 10.1089/jamp.2009.0773
206. Ruivo CF, Adem B, Silva M, Melo SA. The Biology of Cancer Exosomes: Insights and New Perspectives. *Cancer Res* (2017) 77(23):6480–8. doi: 10.1158/0008-5472.CAN-17-0994
207. Bao Q, Gong L, Wang J, Wen J, Shen Y, Zhang W. Extracellular Vesicle RNA Sequencing Reveals Dramatic Transcriptomic Alterations Between Metastatic and Primary Osteosarcoma in a Liquid Biopsy Approach. *Ann Surg Oncol* (2018) 25(9):2642–51. doi: 10.1245/s10434-018-6642-z
208. Jerez S, Araya H, Thaler R, Charlesworth MC, Lopez-Solis R, Kalergis AM, et al. Proteomic Analysis of Exosomes and Exosome-Free Conditioned Media From Human Osteosarcoma Cell Lines Reveals Secretion of Proteins Related to Tumor Progression. *J Cell Biochem* (2017) 118(2):351–60. doi: 10.1002/jcb.25642
209. Kok VC, Yu CC. Cancer-Derived Exosomes: Their Role in Cancer Biology and Biomarker Development. *Int J Nanomed* (2020) 15:8019–36. doi: 10.2147/IJN.S272378
210. Zhao W, Qin P, Zhang D, Cui X, Gao J, Yu Z, et al. Long non-Coding RNA PVT1 Encapsulated in Bone Marrow Mesenchymal Stem Cell-Derived

Exosomes Promotes Osteosarcoma Growth and Metastasis by Stabilizing ERG and Sponging miR-183-5p. *Aging (Albany NY)* (2019) 11(21):9581–96. doi: 10.18632/aging.102406

211. Wang Y, Chu Y, Li K, Zhang G, Guo Z, Wu X, et al. Exosomes Secreted by Adipose-Derived Mesenchymal Stem Cells Foster Metastasis and Osteosarcoma Proliferation by Increasing COLGALT2 Expression. *Front Cell Dev Biol* (2020) 8:353. doi: 10.3389/fcell.2020.00353
212. Liu W, Long Q, Zhang W, Zeng D, Hu B, Liu S, et al. miRNA-221-3p Derived From M2-Polarized Tumor-Associated Macrophage Exosomes Aggravates the Growth and Metastasis of Osteosarcoma Through SOCS3/JAK2/STAT3 Axis. *Aging (Albany NY)* (2021) 13(15):19760–75. doi: 10.18632/aging.203388
213. Zhang H, Yu Y, Wang J, Han Y, Ren T, Huang Y, et al. Macrophages-Derived Exosomal lncRNA LIFR-AS1 Promotes Osteosarcoma Cell Progression *via* miR-29a/NFIA Axis. *Cancer Cell Int* (2021) 21(1):192. doi: 10.1186/s12935-021-01893-0
214. Balkwill F. Cancer and the Chemokine Network. *Nat Rev Cancer* (2004) 4 (7):540–50. doi: 10.1038/nrc1388
215. Li B, Wang Z, Wu H, Xue M, Lin P, Wang S, et al. Epigenetic Regulation of CXCL12 Plays a Critical Role in Mediating Tumor Progression and the Immune Response In Osteosarcoma. *Cancer Res* (2018) 78(14):3938–53. doi: 10.1158/0008-5472.CAN-17-3801
216. Benslimane-Ahmim Z, Pereira J, Lokajczyk A, Dizier B, Galy-Fauroux I, Fischer AM, et al. Osteoprotegerin Regulates Cancer Cell Migration Through SDF-1/CXCR4 Axis and Promotes Tumour Development by Increasing Neovascularization. *Cancer Lett* (2017) 395:11–9. doi: 10.1016/j.canlet.2017.02.032
217. Liao YX, Fu ZZ, Zhou CH, Shan LC, Wang ZY, Yin F, et al. AMD3100 Reduces CXCR4-Mediated Survival and Metastasis of Osteosarcoma by Inhibiting JNK and Akt, But Not P38 or Erk1/2, Pathways in *In Vitro* and Mouse Experiments. *Oncol Rep* (2015) 34(1):33–42. doi: 10.3892/or.2015.3992
218. Georges S, Chesneau J, Hervouet S, Taurelle J, Gouin F, Redini F, et al. A Disintegrin And Metalloproteinase 12 Produced by Tumour Cells Accelerates Osteosarcoma Tumour Progression and Associated Osteolysis. *Eur J Cancer* (2013) 49(9):2253–63. doi: 10.1016/j.ejca.2013.02.020
219. Ory B, Baud'huin M, Verrecchia F, Royer BB, Quillard T, Amiaud J, et al. Blocking HSP90 Addiction Inhibits Tumor Cell Proliferation, Metastasis Development, and Synergistically Acts With Zoledronic Acid to Delay Osteosarcoma Progression. *Clin Cancer Res* (2016) 22(10):2520–33. doi: 10.1158/1078-0432.CCR-15-1925
220. Akiyama T, Dass CR, Choong PF. Novel Therapeutic Strategy for Osteosarcoma Targeting Osteoclast Differentiation, Bone-Resorbing Activity, and Apoptosis Pathway. *Mol Cancer Ther* (2008) 7(11):3461–9. doi: 10.1158/1535-7163.MCT-08-0530
221. Costa-Rodrigues J, Fernandes A, Fernandes MH. Reciprocal Osteoblastic and Osteoclastic Modulation in Co-Cultured MG63 Osteosarcoma Cells and Human Osteoclast Precursors. *J Cell Biochem* (2011) 112(12):3704–13. doi: 10.1002/jcb.23295
222. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and Angiopoietin Signaling in Tumor Angiogenesis and Metastasis. *Trends Mol Med* (2011) 17 (7):347–62. doi: 10.1016/j.molmed.2011.01.015
223. Zetter BR. Angiogenesis and Tumor Metastasis. *Annu Rev Med* (1998) 49:407–24. doi: 10.1146/annurev.med.49.1.407
224. Wang X, Hu Z, Wang Z, Cui Y, Cui X. Angiopoietin-Like Protein 2 is an Important Facilitator of Tumor Proliferation, Metastasis, Angiogenesis and Glycolysis in Osteosarcoma. *Am J Transl Res* (2019) 11(10):6341–55.
225. Wang G, Sun M, Jiang Y, Zhang T, Sun W, Wang H, et al. Anlotinib, a Novel Small Molecular Tyrosine Kinase Inhibitor, Suppresses Growth and Metastasis *via* Dual Blockade of VEGFR2 and MET in Osteosarcoma. *Int J Cancer* (2019) 145(4):979–93. doi: 10.1002/ijc.32180
226. Broadhead ML, Choong PF, Dass CR. Efficacy of Continuously Administered PEDF-Derived Synthetic Peptides Against Osteosarcoma Growth and Metastasis. *J BioMed Biotechnol* (2012) 2012:230298. doi: 10.1155/2012/230298
227. Xie L, Ji T, Guo W. Anti-Angiogenesis Target Therapy for Advanced Osteosarcoma (Review). *Oncol Rep* (2017) 38(2):625–36. doi: 10.3892/or.2017.5735
228. Liu Y, Huang N, Liao S, Rothzerg E, Yao F, Li Y, et al. Current Research Progress in Targeted Anti-Angiogenesis Therapy for Osteosarcoma. *Cell Prolif* (2021) 54(9):e13102. doi: 10.1111/cpr.13102
229. Lehuede C, Dupuy F, Rabinovitch R, Jones RG, Siegel PM. Metabolic Plasticity as a Determinant of Tumor Growth and Metastasis. *Cancer Res* (2016) 76(18):5201–8. doi: 10.1158/0008-5472.CAN-16-0266
230. Giang AH, Raymond T, Brookes P, de Mesy Bentley K, Schwarz E, O'Keefe R, et al. Mitochondrial Dysfunction and Permeability Transition in Osteosarcoma Cells Showing the Warburg Effect. *J Biol Chem* (2013) 288 (46):33303–11. doi: 10.1074/jbc.M113.507129
231. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci* (2016) 41(3):211–8. doi: 10.1016/j.tibs.2015.12.001
232. Hua Y, Qiu Y, Zhao A, Wang X, Chen T, Zhang Z, et al. Dynamic Metabolic Transformation in Tumor Invasion and Metastasis in Mice With LM-8 Osteosarcoma Cell Transplantation. *J Proteome Res* (2011) 10(8):3513–21. doi: 10.1021/pr200147g
233. Roy J, Dibaeinia P, Fan TM, Sinha S, Das A. Global Analysis of Osteosarcoma Lipidomes Reveal Altered Lipid Profiles in Metastatic Versus Nonmetastatic Cells. *J Lipid Res* (2019) 60(2):375–87. doi: 10.1194/jlr.M088559
234. Kort WJ, Hulsmann WC, Stehman TE. Modulation of Metastatic Ability by Inhibition of Cholesterol Synthesis. *Clin Exp Metastasis* (1989) 7(5):517–23. doi: 10.1007/BF01753812
235. Luo X, Cheng C, Tan Z, Li N, Tang M, Yang L, et al. Emerging Roles of Lipid Metabolism in Cancer Metastasis. *Mol Cancer* (2017) 16(1):76. doi: 10.1186/s12943-017-0646-3
236. Ren L, Hong ES, Mendoza A, Issaq S, Tran Hoang C, Lizardo M, et al. Metabolomics Uncovers a Link Between Inositol Metabolism and Osteosarcoma Metastasis. *Oncotarget* (2017) 8(24):38541–53. doi: 10.18632/oncotarget.15872
237. Zhao H, Wu Y, Chen Y, Liu H. Clinical Significance of Hypoxia-Inducible Factor 1 and VEGF-A in Osteosarcoma. *Int J Clin Oncol* (2015) 20(6):1233–43. doi: 10.1007/s10147-015-0848-x
238. Guan G, Zhang Y, Lu Y, Liu L, Shi D, Wen Y, et al. The HIF-1alpha/CXCR4 Pathway Supports Hypoxia-Induced Metastasis of Human Osteosarcoma Cells. *Cancer Lett* (2015) 357(1):254–64. doi: 10.1016/j.canlet.2014.11.034
239. Kunz M, Moeller S, Koczan D, Lorenz P, Wenger RH, Glocker MO, et al. Mechanisms of Hypoxic Gene Regulation of Angiogenesis Factor Cyr61 in Melanoma Cells. *J Biol Chem* (2003) 278(46):45651–60. doi: 10.1074/jbc.M301373200
240. Liapis V, Labrinidis A, Zinonos I, Hay S, Ponomarev V, Panagopoulos V, et al. Hypoxia-Activated Pro-Drug TH-302 Exhibits Potent Tumor Suppressive Activity and Cooperates With Chemotherapy Against Osteosarcoma. *Cancer Lett* (2015) 357(1):160–9. doi: 10.1016/j.canlet.2014.11.020
241. Abu-Remaileh M, Aqeilan RI. Tumor Suppressor WWOX Regulates Glucose Metabolism *via* HIF1alpha Modulation. *Cell Death Differ* (2014) 21 (11):1805–14. doi: 10.1038/cdd.2014.95
242. Gaudio E, Palamarchuk A, Palumbo T, Trapasso F, Pekarsky Y, Croce CM, et al. Physical Association With WWOX Suppresses C-Jun Transcriptional Activity. *Cancer Res* (2006) 66(24):11585–9. doi: 10.1158/0008-5472.CAN-06-3376
243. Del Mare S, Aqeilan RI. Tumor Suppressor WWOX Inhibits Osteosarcoma Metastasis by Modulating RUNX2 Function. *Sci Rep* 5:12959. doi: 10.1038/srep12959
244. Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. Transcription Factors Control Invasion: AP-1 the First Among Equals. *Oncogene* (2007) 26(1):1–10. doi: 10.1038/sj.onc.1209759
245. Leaner VD, Chick JF, Donninger H, Linniola I, Mendoza A, Khanna C, et al. Inhibition of AP-1 Transcriptional Activity Blocks the Migration, Invasion, and Experimental Metastasis of Murine Osteosarcoma. *Am J Pathol* (2009) 174(1):265–75. doi: 10.2353/ajpath.2009.071006
246. Kunita A, Kashima TG, Ohazama A, Grigoriadis AE, Fukayama M. Podoplanin Is Regulated by AP-1 and Promotes Platelet Aggregation and Cell Migration in Osteosarcoma. *Am J Pathol* (2011) 179(2):1041–9. doi: 10.1016/j.ajpath.2011.04.027
247. Weekes D, Kashima TG, Zandueta C, Perurena N, Thomas DP, Sunters A, et al. Regulation of Osteosarcoma Cell Lung Metastasis by the C-Fos/AP-1 Target FGFR1. *Oncogene* (2016) 35(22):2852–61. doi: 10.1038/onc.2015.344

248. Chano T, Avnet S, Kusuzaki K, Bonuccelli G, Sonveaux P, Rotili D, et al. Tumour-Specific Metabolic Adaptation to Acidosis is Coupled to Epigenetic Stability in Osteosarcoma Cells. *Am J Cancer Res* (2016) 6(4):859–75.
249. Avnet S, Di Pompo G, Chano T, Errani C, Ibrahim-Hashim A, Gillies RJ, et al. Cancer-Associated Mesenchymal Stroma Fosters the Stemness of Osteosarcoma Cells in Response to Intratumoral Acidosis *via* NF-kappaB Activation. *Int J Cancer* (2017) 140(6):1331–45. doi: 10.1002/ijc.30540
250. Avnet S, Chano T, Massa A, Bonuccelli G, Lemma S, Falzetti L, et al. Acid Microenvironment Promotes Cell Survival of Human Bone Sarcoma Through the Activation of cIAP Proteins and NF-kappaB Pathway. *Am J Cancer Res* (2019) 9(6):1127–44.
251. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial Oxidative Stress: Implications for Cell Death. *Annu Rev Pharmacol Toxicol* (2007) 47:143–83. doi: 10.1146/annurev.pharmtox.47.120505.105122
252. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, Oxidative Stress and Cell Death. *Apoptosis* (2007) 12(5):913–22. doi: 10.1007/s10495-007-0756-2
253. Qiu H, Orr FW, Jensen D, Wang HH, McIntosh AR, Hasinoff BB, et al. Arrest of B16 Melanoma Cells in the Mouse Pulmonary Microcirculation Induces Endothelial Nitric Oxide Synthase-Dependent Nitric Oxide Release That Is Cytotoxic to the Tumor Cells. *Am J Pathol* (2003) 162(2):403–12. doi: 10.1016/S0002-9440(10)63835-7
254. Poderoso JJ, Helfenberger K, Poderoso C. The Effect of Nitric Oxide on Mitochondrial Respiration. *Nitric Oxide* (2019) 88:61–72. doi: 10.1016/j.niox.2019.04.005
255. Ridnour LA, Thomas DD, Mancardi D, Espey MG, Miranda KM, Paolocci N, et al. The Chemistry of Nitrosative Stress Induced by Nitric Oxide and Reactive Nitrogen Oxide Species. Putting Perspective on Stressful Biological Situations. *Biol Chem* (2004) 385(1):1–10. doi: 10.1515/BC.2004.001
256. Winterbourn CC. Revisiting the Reactions of Superoxide With Glutathione and Other Thiols. *Arch Biochem Biophys* (2016) 595:68–71. doi: 10.1016/j.abb.2015.11.028
257. Buddingh EP, Kuijjer ML, Duim RA, Burger H, Agelopoulos K, Myklebost O, et al. Tumor-Infiltrating Macrophages are Associated With Metastasis Suppression in High-Grade Osteosarcoma: A Rationale for Treatment With Macrophage Activating Agents. *Clin Cancer Res* (2011) 17(8):2110–9. doi: 10.1158/1078-0432.CCR-10-2047
258. Dumars C, Ngyuen JM, Gaultier A, Lanel R, Corradini N, Gouin F, et al. Dysregulation of Macrophage Polarization is Associated With the Metastatic Process in Osteosarcoma. *Oncotarget* (2016) 7(48):78343–54. doi: 10.18632/oncotarget.13055
259. Han Q, Shi H, Liu F. CD163(+) M2-Type Tumor-Associated Macrophage Support the Suppression of Tumor-Infiltrating T Cells in Osteosarcoma. *Int Immunopharmacol* (2016) 34:101–6. doi: 10.1016/j.intimp.2016.01.023
260. Hingorani P, Maas ML, Gustafson MP, Dickman P, Adams RH, Watanabe M, et al. Increased CTLA-4(+) T Cells and an Increased Ratio of Monocytes With Loss of Class II (CD14(+) HLA-DR(lo/neg)) Found in Aggressive Pediatric Sarcoma Patients. *J Immunother Cancer* 3:35. doi: 10.1186/s40425-015-0082-0
261. Gomez-Brouchet A, Illac C, Gilhodes J, Bouvier C, Aubert S, Guinebretiere JM, et al. CD163-Positive Tumor-Associated Macrophages and CD8-Positive Cytotoxic Lymphocytes are Powerful Diagnostic Markers for the Therapeutic Stratification of Osteosarcoma Patients: An Immunohistochemical Analysis of the Biopsies Fromthe French OS2006 Phase 3 Trial. *Oncoimmunology* (2017) 6(9):e1331193. doi: 10.1080/2162402X.2017.1331193
262. Pahl JH, Kwappenberg KM, Varypataki EM, Santos SJ, Kuijjer ML, Mohamed S, et al. Macrophages Inhibit Human Osteosarcoma Cell Growth After Activation With the Bacterial Cell Wall Derivative Liposomal Muramyl Tripeptide in Combination With Interferon-Gamma. *J Exp Clin Cancer Res* (2014) 33:27. doi: 10.1186/1756-9966-33-27
263. Shao XJ, Xiang SF, Chen YQ, Zhang N, Cao J, Zhu H, et al. Inhibition of M2-Like Macrophages by All-Trans Retinoic Acid Prevents Cancer Initiation and Stemness in Osteosarcoma Cells. *Acta Pharmacol Sin* (2019) 40(10):1343–50. doi: 10.1038/s41401-019-0262-4
264. Zhou Q, Xian M, Xiang S, Xiang D, Shao X, Wang J, et al. All-Trans Retinoic Acid Prevents Osteosarcoma Metastasis by Inhibiting M2 Polarization of Tumor-Associated Macrophages. *Cancer Immunol Res* (2017) 5(7):547–59. doi: 10.1158/2326-6066.CIR-16-0259
265. Koirala P, Roth ME, Gill J, Piperdi S, Chinai JM, Geller DS, et al. Immune Infiltration and PD-L1 Expression in the Tumor Microenvironment are Prognostic in Osteosarcoma. *Sci Rep* (2016) 6:30093. doi: 10.1038/srep30093
266. Fritzsching B, Fellenberg J, Moskovszky L, Sapi Z, Krenacs T, Machado I, et al. CD8(+)/FOXP3(+)-Ratio in Osteosarcoma Microenvironment Separates Survivors From Non-Survivors: A Multicenter Validated Retrospective Study. *Oncoimmunology* (2015) 4(3):e990800. doi: 10.4161/2162402X.2014.990800
267. Sundara YT, Kostine M, Cleven AH, Bovee JV, Schilham MW, Cleton-Jansen AM. Increased PD-L1 and T-Cell Infiltration in the Presence of HLA Class I Expression in Metastatic High-Grade Osteosarcoma: A Rationale for T-Cell-Based Immunotherapy. *Cancer Immunol Immunother* (2017) 66(1):119–28. doi: 10.1007/s00262-016-1925-3
268. Chen C, Xie L, Ren T, Huang Y, Xu J, Guo W. Immunotherapy for Osteosarcoma: Fundamental Mechanism, Rationale, and Recent Breakthroughs. *Cancer Lett* (2021) 500:1–10. doi: 10.1016/j.canlet.2020.12.024
269. Yang JX, Rastetter RH, Wilhelm D. Non-Coding RNAs: An Introduction. *Adv Exp Med Biol* (2016) 886:13–32. doi: 10.1007/978-94-017-7417-8_2
270. Gao Y, Luo LH, Li S, Yang C. miR-17 Inhibitor Suppressed Osteosarcoma Tumor Growth and Metastasis *via* Increasing PTEN Expression. *Biochem Biophys Res Commun* (2014) 444(2):230–4. doi: 10.1016/j.bbrc.2014.01.061
271. Ding J, Sha L, Shen P, Huang M, Cai Q, Li J. MicroRNA-18a Inhibits Cell Growth and Induces Apoptosis in Osteosarcoma by Targeting MED27. *Int J Oncol* (2018) 53(1):329–38. doi: 10.3892/ijo.2018.4374
272. Sun Z, Liu Q, Hong H, Zhang H, Zhang T. miR-19 Promotes Osteosarcoma Progression by Targeting SOCS6. *Biochem Biophys Res Commun* (2018) 495(1):1363–9. doi: 10.1016/j.bbrc.2017.10.002
273. Xin M, Qiao Z, Li J, Liu J, Song S, Zhao X, et al. miR-22 Inhibits Tumor Growth and Metastasis by Targeting ATP Citrate Lyase: Evidence in Osteosarcoma, Prostate Cancer, Cervical Cancer and Lung Cancer. *Oncotarget* (2016) 7(28):44252–65. doi: 10.18632/oncotarget.10020
274. He Y, Meng C, Shao Z, Wang H, Yang S. MiR-23a Functions as a Tumor Suppressor in Osteosarcoma. *Cell Physiol Biochem* (2014) 34(5):1485–96. doi: 10.1159/000366353
275. Chen B, Liu J, Qu J, Song Y, Li Y, Pan S. MicroRNA-25 Suppresses Proliferation, Migration, and Invasion of Osteosarcoma by Targeting SOX4. *Tumour Biol* (2017) 39(7):1010428317703841. doi: 10.1177/1010428317703841
276. Lu J, Song G, Tang Q, Yin J, Zou C, Zhao Z, et al. MiR-26a Inhibits Stem Cell-Like Phenotype and Tumor Growth of Osteosarcoma by Targeting Jagged1. *Oncogene* (2017) 36(2):231–41. doi: 10.1038/onc.2016.194
277. Zhang R, Yan S, Wang J, Deng F, Guo Y, Li Y, et al. MiR-30a Regulates the Proliferation, Migration, and Invasion of Human Osteosarcoma by Targeting Runx2. *Tumour Biol* (2016) 37(3):3479–88. doi: 10.1007/s13277-015-4086-7
278. Tao J, Cong H, Wang H, Zhang D, Liu C, Chu H, et al. MiR-30a-5p Inhibits Osteosarcoma Cell Proliferation and Migration by Targeting FOXD1. *Biochem Biophys Res Commun* (2018) 503(2):1092–7. doi: 10.1016/j.bbrc.2018.06.121
279. Zhao Y, Tu MJ, Yu YF, Wang WP, Chen QX, Qiu JX, et al. Combination Therapy With Bioengineered miR-34a Prodrug and Doxorubicin Synergistically Suppresses Osteosarcoma Growth. *Biochem Pharmacol* (2015) 98(4):602–13. doi: 10.1016/j.bcp.2015.10.015
280. Liu Q, Song Y, Duan X, Chang Y, Guo J. MiR-92a Inhibits the Progress of Osteosarcoma Cells and Increases the Cisplatin Sensitivity by Targeting Notch1. *BioMed Res Int* (2018) 2018:9870693. doi: 10.1155/2018/9870693
281. Yu B, Jiang K, Zhang J. MicroRNA-124 Suppresses Growth and Aggressiveness of Osteosarcoma and Inhibits TGF-Beta-Mediated AKT/GSK-3beta/SNAIL-1 Signaling. *Mol Med Rep* (2018) 17(5):6736–44. doi: 10.3892/mmr.2018.8637
282. Liu LH, Li H, Li JP, Zhong H, Zhang HC, Chen J, et al. miR-125b Suppresses the Proliferation and Migration of Osteosarcoma Cells Through Down-Regulation of STAT3. *Biochem Biophys Res Commun* (2011) 416(1-2):31–8. doi: 10.1016/j.bbrc.2011.10.117

283. Bao Y, Peng L, Ma J, Liu K, Li W. Decreased miR-134 Expression and its Tumor-Suppressive Function in Human Osteosarcoma. *Genet Mol Res* (2015) 14(4):16771–81. doi: 10.4238/2015.December.14.4
284. Li ZM, Zhang HY, Wang YX, Wang WB. MicroRNA-137 Is Downregulated in Human Osteosarcoma and Regulates Cell Proliferation and Migration Through Targeting FXYD6. *J Drug Target* (2016) 24(2):102–10. doi: 10.3109/1061186X.2015.1057149
285. Shi YK, Guo YH. MiR-139-5p Suppresses Osteosarcoma Cell Growth and Invasion Through Regulating DNMT1. *Biochem Biophys Res Commun* (2018) 503(2):459–66. doi: 10.1016/j.bbrc.2018.04.124
286. Gu R, Sun YF, Wu MF, Liu JB, Jiang JL, Wang SH, et al. Biological Roles of microRNA-140 in Tumor Growth, Migration, and Metastasis of Osteosarcoma *In Vivo* and In Vitro. *Tumour Biol* (2016) 37(1):353–60. doi: 10.1007/s13277-015-3801-8
287. Xiao Q, Huang L, Zhang Z, Chen X, Luo J, Zhang Z, et al. Overexpression of miR-140 Inhibits Proliferation of Osteosarcoma Cells *via* Suppression of Histone Deacetylase 4. *Oncol Res* (2017) 25(2):267–75. doi: 10.3727/096504016X14732510786564
288. Wang W, Zhou X, Wei M. MicroRNA-144 Suppresses Osteosarcoma Growth and Metastasis by Targeting ROCK1 and ROCK2. *Oncotarget* (2015) 6(12):10297–308. doi: 10.18632/oncotarget.3305
289. Li Y, Liu J, Liu ZZ, Wei WB. MicroRNA-145 Inhibits Tumour Growth and Metastasis in Osteosarcoma by Targeting Cyclin-Dependent Kinase, CDK6. *Eur Rev Med Pharmacol Sci* (2016) 20(24):5117–25.
290. Yang H, Peng Z, Da Z, Li X, Cheng Y, Tan B, et al. MicroRNA-148a Acts as a Tumor Suppressor in Osteosarcoma *via* Targeting Rho-Associated Coiled-Coil Kinase. *Oncol Res* (2017) 25(8):1231–43. doi: 10.3727/096504017X14850134190255
291. Zhou H, Zhang M, Yuan H, Zheng W, Meng C, Zhao D. MicroRNA-154 Functions as a Tumor Suppressor in Osteosarcoma by Targeting Wnt5a. *Oncol Rep* (2016) 35(3):1851–8. doi: 10.3892/or.2015.4495
292. Jiang C, Fang X, Zhang H, Wang X, Li M, Jiang W, et al. Triptolide Inhibits the Growth of Osteosarcoma by Regulating microRNA-181a *via* Targeting PTEN Gene *In Vivo* and Vitro. *Tumour Biol* (2017) 39(4):1010428317697556. doi: 10.1177/1010428317697556
293. Zhang Z, Zhang W, Mao J, Xu Z, Fan M. miR-186-5p Functions as a Tumor Suppressor in Human Osteosarcoma by Targeting Foxk1. *Cell Physiol Biochem* (2019) 52(3):553–64. doi: 10.33594/000000039
294. Pan L, Meng L, Liang F, Cao L. Mir188 Suppresses Tumor Progression by Targeting SOX4 in Pediatric Osteosarcoma. *Mol Med Rep* (2018) 18(1):441–6. doi: 10.3892/mmr.2018.8997
295. Pu Y, Zhao F, Cai W, Meng X, Li Y, Cai S. MiR-193a-3p and miR-193a-5p Suppress the Metastasis of Human Osteosarcoma Cells by Down-Regulating Rab27B and SRR, Respectively. *Clin Exp Metastasis* (2016) 33(4):359–72. doi: 10.1007/s10585-016-9783-0
296. Li M, Shen Y, Wang Q, Zhou X. MiR-204-5p Promotes Apoptosis and Inhibits Migration of Osteosarcoma *via* Targeting EBF2. *Biochimie* (2019) 158:224–32. doi: 10.1016/j.biochi.2018.12.003
297. Jiang Z, Jiang C, Yu C, Fang J. MicroRNA-208b Inhibits Human Osteosarcoma Progression by Targeting ROR2. *Tumour Biol* (2017) 39(6):1010428317705751. doi: 10.1177/1010428317705751
298. Liu C, Tang X. Downregulation of microRNA-210 Inhibits Osteosarcoma Growth *In Vitro* and In Vivo. *Mol Med Rep* (2015) 12(3):3674–80. doi: 10.3892/mmr.2015.3880
299. Luo XJ, Tang DG, Gao TL, Zhang YL, Wang M, Quan ZX, et al. MicroRNA-212 Inhibits Osteosarcoma Cells Proliferation and Invasion by Down-Regulation of Sox4. *Cell Physiol Biochem* (2014) 34(6):2180–8. doi: 10.1159/000369661
300. Xu Z, Wang T. miR-214 Promotes the Proliferation and Invasion of Osteosarcoma Cells Through Direct Suppression of LZTS1. *Biochem Biophys Res Commun* (2014) 449(2):190–5. doi: 10.1016/j.bbrc.2014.04.140
301. Sun B, Yang M, Li M, Wang F. The microRNA-217 Functions as a Tumor Suppressor and is Frequently Downregulated in Human Osteosarcoma. *BioMed Pharmacother* (2015) 71:58–63. doi: 10.1016/j.biopha.2015.02.014
302. Jiang W, Liu J, Xu T, Yu X. MiR-329 Suppresses Osteosarcoma Development by Downregulating Rab10. *FEBS Lett* (2016) 590(17):2973–81. doi: 10.1002/1873-3468.12337
303. He F, Fang L, Yin Q. miR-363 Acts as a Tumor Suppressor in Osteosarcoma Cells by Inhibiting PDZD2. *Oncol Rep* (2019) 41(5):2729–38. doi: 10.3892/or.2019.7078
304. Xu SY, Xu PF, Gao TT. MiR-372-3p Inhibits the Growth and Metastasis of Osteosarcoma Cells by Targeting FXYD6. *Eur Rev Med Pharmacol Sci* (2018) 22(1):62–9. doi: 10.26355/eurrev_201801_14101
305. Li Z, Shen J, Chan MT, Wu WK. MicroRNA-379 Suppresses Osteosarcoma Progression by Targeting PDK1. *J Cell Mol Med* (2017) 21(2):315–23. doi: 10.1111/jcmm.12966
306. Zhao D, Jia P, Wang W, Zhang G. VEGF-Mediated Suppression of Cell Proliferation and Invasion by miR-410 in Osteosarcoma. *Mol Cell Biochem* 400(1-2):87–95. doi: 10.1007/s11010-014-2265-2
307. Yang G, Zhang C, Wang N, Chen J. miR-425-5p Decreases LncRNA MALAT1 and TUG1 Expressions and Suppresses Tumorigenesis in Osteosarcoma *via* Wnt/beta-Catenin Signaling Pathway. *Int J Biochem Cell Biol* (2019) 111:42–51. doi: 10.1016/j.biocel.2019.04.004
308. Yuan J, Lang J, Liu C, Zhou K, Chen L, Liu Y. The Expression and Function of miRNA-451 in Osteosarcoma. *Med Oncol* (2015) 32(1):324. doi: 10.1007/s12032-014-0324-x
309. Yuan W, Wang D, Liu Y, Tian D, Wang Y, Zhang R, et al. Mir494 Inhibits Cell Proliferation and Metastasis *via* Targeting of CDK6 in Osteosarcoma. *Mol Med Rep* (2017) 16(6):8627–34. doi: 10.3892/mmr.2017.7709
310. Qi NN, Tian S, Li X, Wang FL, Liu B. Up-Regulation of microRNA-496 Suppresses Proliferation, Invasion, Migration and *In Vivo* Tumorigenicity of Human Osteosarcoma Cells by Targeting Eif4e. *Biochimie* (2019) 163:1–11. doi: 10.1016/j.biochi.2019.04.017
311. Pang PC, Shi XY, Huang WL, Sun K. miR-497 as a Potential Serum Biomarker for the Diagnosis and Prognosis of Osteosarcoma. *Eur Rev Med Pharmacol Sci* (2016) 20(18):3765–9.
312. Cai W, Xu Y, Yin J, Zuo W, Su Z. Mir5905p Suppresses Osteosarcoma Cell Proliferation and Invasion *via* Targeting KLF5. *Mol Med Rep* (2018) 18(2):2328–34. doi: 10.3892/mmr.2018.9173
313. Liu K, Sun X, Zhang Y, Liu L, Yuan Q. MiR-598: A Tumor Suppressor With Biomarker Significance in Osteosarcoma. *Life Sci* (2017) 188:141–8. doi: 10.1016/j.lfs.2017.09.003
314. Ma C, Zhan C, Yuan H, Cui Y, Zhang Z. MicroRNA-603 Functions as an Oncogene by Suppressing BRCC2 Protein Translation in Osteosarcoma. *Oncol Rep* (2016) 35(6):3257–64. doi: 10.3892/or.2016.4718
315. Wang H, Xing D, Ren D, Feng W, Chen Y, Zhao Z, et al. MicroRNA643 Regulates the Expression of ZEB1 and Inhibits Tumorigenesis in Osteosarcoma. *Mol Med Rep* (2017) 16(4):5157–64. doi: 10.3892/mmr.2017.7273
316. Zhang L, Wang Y, Zhang L, Xia X, Chao Y, He R, et al. ZBTB7A, a miR-663a Target Gene, Protects Osteosarcoma From Endoplasmic Reticulum Stress-Induced Apoptosis by Suppressing LncRNA GAS5 Expression. *Cancer Lett* (2019) 448:105–16. doi: 10.1016/j.canlet.2019.01.046
317. Liu Y, Wang Y, Yang H, Zhao L, Song R, Tan H, et al. MicroRNA873 Targets HOXA9 to Inhibit the Aggressive Phenotype of Osteosarcoma by Deactivating the Wnt/betacatenin Pathway. *Int J Oncol* (2019) 54(5):1809–20. doi: 10.3892/ijo.2019.4735
318. Ghosh T, Varshney A, Kumar P, Kaur M, Kumar V, Shekhar R, et al. MicroRNA-874-Mediated Inhibition of the Major G1/S Phase Cyclin, CCNE1, is Lost in Osteosarcomas. *J Biol Chem* (2017) 292(52):21264–81. doi: 10.1074/jbc.M117.808287
319. Zhong L, Zheng C, Fang H, Xu M, Chen B, Li C. MicroRNA-1270 is Associated With Poor Prognosis and its Inhibition Yielded Anticancer Mechanisms in Human Osteosarcoma. *IUBMB Life* (2018) 70(7):625–32. doi: 10.1002/iub.1753
320. Yuan H, Gao Y. MicroRNA-1908 is Upregulated in Human Osteosarcoma and Regulates Cell Proliferation and Migration by Repressing PTEN Expression. *Oncol Rep* (2015) 34(5):2706–14. doi: 10.3892/or.2015.4242
321. Shi D, Wu F, Mu S, Hu B, Zhong B, Gao F, et al. LncRNA AFAP1-AS1 Promotes Tumorigenesis and Epithelial-Mesenchymal Transition of Osteosarcoma Through RhoC/ROCK1/p38MAPK/Twist1 Signaling Pathway. *J Exp Clin Cancer Res* (2019) 38(1):375. doi: 10.1186/s13046-019-1363-0
322. Li R, Liu S, Li Y, Tang Q, Xie Y, Zhai R. Long Noncoding RNA AFAP1AS1 Enhances Cell Proliferation and Invasion in Osteosarcoma Through

Regulating Mir46955p/TCF4betacatenin Signaling. *Mol Med Rep* (2018) 18 (2):1616–22. doi: 10.3892/mmr.2018.9131
323. Lu L, Dai Z, Luo Q, Lv G. The Long Noncoding RNA Cancer Susceptibility Candidate 2 Inhibits Tumor Progression in Osteosarcoma. *Mol Med Rep* (2018) 17(1):1947–53. doi: 10.3892/mmr.2017.8080
324. Zhao W, Zhang D, Qin P, Zhang J, Cui X, Gao J, et al. Long non-Coding RNA EPIC1 Inhibits Viability and Invasion of Osteosarcoma Cells by Promoting MEF2D Ubiquitylation. *Int J Biol Macromol* (2019) 128:566–73. doi: 10.1016/j.ijbiomac.2019.01.156
325. Kun-Peng Z, Chun-Lin Z, Xiao-Long M. Antisense lncRNA FOXF1-AS1 Promotes Migration and Invasion of Osteosarcoma Cells Through the FOXF1/MMP-2/-9 Pathway. *Int J Biol Sci* (2017) 13(9):1180–91. doi: 10.7150/ijbs.21722
326. Sun J, Wang X, Fu C, Wang X, Zou J, Hua H, et al. Long Noncoding RNA FGFR3-AS1 Promotes Osteosarcoma Growth Through Regulating its Natural Antisense Transcript FGFR3. *Mol Biol Rep* (2016) 43(5):427–36. doi: 10.1007/s11033-016-3975-1
327. Ren Z, Hu Y, Li G, Kang Y, Liu Y, Zhao H. HIF-1alpha Induced Long Noncoding RNA FOXD2-AS1 Promotes the Osteosarcoma Through Repressing P21. *BioMed Pharmacother* (2019) 117:109104. doi: 10.1016/j.biopha.2019.109104
328. Ye K, Wang S, Zhang H, Han H, Ma B, Nan W. Long Noncoding RNA GAS5 Suppresses Cell Growth and Epithelial-Mesenchymal Transition in Osteosarcoma by Regulating the miR-221/ARHI Pathway. *J Cell Biochem* (2017) 118(12):4772–81. doi: 10.1002/jcb.26145
329. Qu Y, Zheng S, Kang M, Dong R, Zhou H, Zhao D, et al. Knockdown of Long non-Coding RNA HOXD-AS1 Inhibits the Progression of Osteosarcoma. *BioMed Pharmacother* (2018) 98:899–906. doi: 10.1016/j.biopha.2018.01.024
330. Wang B, Su Y, Yang Q, Lv D, Zhang W, Tang K, et al. Overexpression of Long Non-Coding RNA HOTAIR Promotes Tumor Growth and Metastasis in Human Osteosarcoma. *Mol Cells* (2015) 38(5):432–40. doi: 10.14348/molcells.2015.2327
331. Gu Z, Hou Z, Zheng L, Wang X, Wu L, Zhang C. Long Noncoding RNA LINC00858 Promotes Osteosarcoma Through Regulating miR-139-CDK14 Axis. *Biochem Biophys Res Commun* (2018) 503(2):1134–40. doi: 10.1016/j.bbrc.2018.06.131
332. Zhang B, Yu L, Han N, Hu Z, Wang S, Ding L, et al. LINC01116 Targets miR-520a-3p and Affects IL6R to Promote the Proliferation and Migration of Osteosarcoma Cells Through the Jak-Stat Signaling Pathway. *BioMed Pharmacother* (2018) 107:270–82. doi: 10.1016/j.biopha.2018.07.119
333. Han Z, Shi L. Long non-Coding RNA LUCAT1 Modulates Methotrexate Resistance in Osteosarcoma *via* miR-200c/ABCB1 Axis. *Biochem Biophys Res Commun* (2018) 495(1):947–53. doi: 10.1016/j.bbrc.2017.11.121
334. Chen Y, Huang W, Sun W, Zheng B, Wang C, Luo Z, et al. LncRNA MALAT1 Promotes Cancer Metastasis in Osteosarcoma *via* Activation of the PI3K-Akt Signaling Pathway. *Cell Physiol Biochem* (2018) 51(3):1313–26. doi: 10.1159/000495550
335. Dong Y, Liang G, Yuan B, Yang C, Gao R, Zhou X. MALAT1 Promotes the Proliferation and Metastasis of Osteosarcoma Cells by Activating the PI3K/Akt Pathway. *Tumour Biol* (2015) 36(3):1477–86. doi: 10.1007/s13277-014-2631-4
336. Duan G, Zhang C, Xu C, Xu C, Zhang L, Zhang Y. Knockdown of MALAT1 Inhibits Osteosarcoma Progression *via* Regulating the Mir34a/Cyclin D1 Axis. *Int J Oncol* (2019) 54(1):17–28. doi: 10.3892/ijo.2018.4600
337. Li J, Wu QM, Wang XQ, Zhang CQ. Long Noncoding RNA Mir210hg Sponges miR-503 to Facilitate Osteosarcoma Cell Invasion and Metastasis. *DNA Cell Biol* (2017) 36(12):1117–25. doi: 10.1089/dna.2017.3888
338. Hu Y, Yang Q, Wang L, Wang S, Sun F, Xu D, et al. Knockdown of the Oncogene lncRNA NEAT1 Restores the Availability of miR-34c and Improves the Sensitivity to Cisplatin in Osteosarcoma. *Biosci Rep* (2018) 38(3):BSR20180375. doi: 10.1042/BSR20180375
339. Ye H, Lin J, Yao X, Li Y, Lin X, Lu H. Overexpression of Long Non-Coding RNA NNT-AS1 Correlates With Tumor Progression and Poor Prognosis in Osteosarcoma. *Cell Physiol Biochem* (2018) 45(5):1904–14. doi: 10.1159/000487966
340. Zhu KP, Ma XL, Zhang CL. LncRNA ODRUL Contributes to Osteosarcoma Progression Through the miR-3182/MMP2 Axis. *Mol Ther* (2017) 25 (10):2383–93. doi: 10.1016/j.ymthe.2017.06.027
341. Wang J, Cao L, Wu J, Wang Q. Long non-Coding RNA SNHG1 Regulates NOB1 Expression by Sponging miR-326 and Promotes Tumorigenesis in Osteosarcoma. *Int J Oncol* (2018) 52(1):77–88. doi: 10.3892/ijo.2017.4187
342. Jiang Z, Jiang C, Fang J. Up-Regulated lnc-SNHG1 Contributes to Osteosarcoma Progression Through Sequestration of miR-577 and Activation of WNT2B/Wnt/beta-Catenin Pathway. *Biochem Biophys Res Commun* (2018) 495(1):238–45. doi: 10.1016/j.bbrc.2017.11.012
343. Deng Y, Zhao F, Zhang Z, Sun F, Wang M. Long Noncoding RNA SNHG7 Promotes the Tumor Growth and Epithelial-To-Mesenchymal Transition *via* Regulation of miR-34a Signals in Osteosarcoma. *Cancer Biother Radiopharm* (2018) 33(9):365–72. doi: 10.1089/cbr.2018.2503
344. Yang G, Song R, Wang L, Wu X. Knockdown of Long non-Coding RNA TP73-AS1 Inhibits Osteosarcoma Cell Proliferation and Invasion Through Sponging miR-142. *BioMed Pharmacother* (2018) 103:1238–45. doi: 10.1016/j.biopha.2018.04.146
345. Wang H, Yu Y, Fan S, Luo L. Knockdown of Long Noncoding RNA TUG1 Inhibits the Proliferation and Cellular Invasion of Osteosarcoma Cells by Sponging miR-153. *Oncol Res* (2018) 26(5):665–73. doi: 10.3727/096504017X14908298412505
346. Yang C, Wu K, Wang S, Wei G. Long non-Coding RNA XIST Promotes Osteosarcoma Progression by Targeting YAP *via* miR-195-5p. *J Cell Biochem* (2018) 119(7):5646–56. doi: 10.1002/jcb.26743
347. Zhang R, Xia T. Long non-Coding RNA XIST Regulates PDCD4 Expression by Interacting With miR-21-5p and Inhibits Osteosarcoma Cell Growth and Metastasis. *Int J Oncol* (2017) 51(5):1460–70. doi: 10.3892/ijo.2017.4127
348. Liu G, Huang K, Jie Z, Wu Y, Chen J, Chen Z, et al. CircFAT1 Sponges miR-375 to Promote the Expression of Yes-Associated Protein 1 in Osteosarcoma Cells. *Mol Cancer* (2018) 17(1):170. doi: 10.1186/s12943-018-0917-7
349. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* (2004) 116(2):281–97. doi: 10.1016/s0092-8674(04)00045-5
350. Pasquinelli AE, Hunter S, Bracht J. MicroRNAs: A Developing Story. *Curr Opin Genet Dev* (2005) 15(2):200–5. doi: 10.1016/j.gde.2005.01.002
351. Kim VN, Nam JW. Genomics of microRNA. *Trends Genet* (2006) 22(3):165–73. doi: 10.1016/j.tig.2006.01.003
352. Mao JH, Zhou RP, Peng AF, Liu ZL, Huang SH, Long XH, et al. microRNA-195 Suppresses Osteosarcoma Cell Invasion and Migration *In Vitro* by Targeting FASN. *Oncol Lett* (2012) 4(5):1125–9. doi: 10.3892/ol.2012.863
353. Cai H, Zhao H, Tang J, Wu H. Serum miR-195 is a Diagnostic and Prognostic Marker for Osteosarcoma. *J Surg Res* (2015) 194(2):505–10. doi: 10.1016/j.jss.2014.11.025
354. Lian F, Cui Y, Zhou C, Gao K, Wu L. Identification of a Plasma four-microRNA Panel as Potential Noninvasive Biomarker for Osteosarcoma. *PloS One* (2015) 10(3):e0121499. doi: 10.1371/journal.pone.0121499
355. Salah Z, Arafeh R, Maximov V, Galasso M, Khawaled S, Abou-Sharieha S, et al. miR-27a and miR-27a* Contribute to Metastatic Properties of Osteosarcoma Cells. *Oncotarget* (2015) 6(7):4920–35. doi: 10.18632/oncotarget.3025
356. Jones KB, Salah Z, Del Mare S, Galasso M, Gaudio E, Nuovo GJ, et al. miRNA Signatures Associate With Pathogenesis and Progression of Osteosarcoma. *Cancer Res* (2012) 72(7):1865–77. doi: 10.1158/0008-5472.CAN-11-2663
357. Pan W, Wang H, Jianwei R, Ye Z. MicroRNA-27a Promotes Proliferation, Migration and Invasion by Targeting MAP2K4 in Human Osteosarcoma Cells. *Cell Physiol Biochem* (2014) 33(2):402–12. doi: 10.1159/000356679
358. Dai N, Zhong ZY, Cun YP, Qing Y, Chen C, Jiang P, et al. Alteration of the microRNA Expression Profile in Human Osteosarcoma Cells Transfected With APE1 siRNA. *Neoplasma* (2013) 60(4):384–94. doi: 10.4149/neo_2013_050
359. Zhang Y, Hu H, Song L, Cai L, Wei R, Jin W. Epirubicin-Mediated Expression of miR-302b is Involved in Osteosarcoma Apoptosis and Cell Cycle Regulation. *Toxicol Lett* (2013) 222(1):1–9. doi: 10.1016/j.toxlet.2013.06.242
360. Li Y, Zhang J, Zhang L, Si M, Yin H, Li J. Diallyl Trisulfide Inhibits Proliferation, Invasion and Angiogenesis of Osteosarcoma Cells by Switching on Suppressor microRNAs and Inactivating of Notch-1 Signaling. *Carcinogenesis* (2013) 34(7):1601–10. doi: 10.1093/carcin/bgt065
361. Chen Y, Zhu X, Zhang X, Liu B, Huang L. Nanoparticles Modified With Tumor-Targeting scFv Deliver siRNA and miRNA for Cancer Therapy. *Mol Ther* (2010) 18(9):1650–6. doi: 10.1038/mt.2010.136

362. Varshney J, Subramanian S. MicroRNAs as Potential Target in Human Bone and Soft Tissue Sarcoma Therapeutics. *Front Mol Biosci* (2015) 2:31. doi: 10.3389/fmolb.2015.00031
363. Qi P, Du X. The Long non-Coding RNAs, a New Cancer Diagnostic and Therapeutic Gold Mine. *Mod Pathol* (2013) 26(2):155–65. doi: 10.1038/modpathol.2012.160
364. Yang Z, Li X, Yang Y, He Z, Qu X, Zhang Y. Long Noncoding RNAs in the Progression, Metastasis, and Prognosis of Osteosarcoma. *Cell Death Dis* (2016) 7(9):e2389. doi: 10.1038/cddis.2016.272
365. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, et al. MALAT-1, a Novel Noncoding RNA, and Thymosin Beta4 Predict Metastasis and Survival in Early-Stage non-Small Cell Lung Cancer. *Oncogene* (2003) 22(39):8031–41. doi: 10.1038/sj.onc.1206928
366. Gao KT, Lian D. Long non-Coding RNA MALAT1 is an Independent Prognostic Factor of Osteosarcoma. *Eur Rev Med Pharmacol Sci* (2016) 20 (17):3561–5.
367. Zhu L, Liu J, Ma S, Zhang S. Long Noncoding RNA MALAT-1 Can Predict Metastasis and a Poor Prognosis: A Meta-Analysis. *Pathol Oncol Res* (2015) 21(4):1259–64. doi: 10.1007/s12253-015-9960-5
368. Liu M, Yang P, Mao G, Deng J, Peng G, Ning X, et al. Long non-Coding RNA MALAT1 as a Valuable Biomarker for Prognosis in Osteosarcoma: A Systematic Review and Meta-Analysis. *Int J Surg* (2019) 72:206–13. doi: 10.1016/j.ijsu.2019.11.004
369. Huo Y, Li Q, Wang X, Jiao X, Zheng J, Li Z, et al. MALAT1 Predicts Poor Survival in Osteosarcoma Patients and Promotes Cell Metastasis Through Associating With EZH2. *Oncotarget* (2017) 8(29):46993–7006. doi: 10.18632/oncotarget.16551
370. Fang D, Yang H, Lin J, Teng Y, Jiang Y, Chen J, et al. 17beta-Estradiol Regulates Cell Proliferation, Colony Formation, Migration, Invasion and Promotes Apoptosis by Upregulating miR-9 and Thus Degrades MALAT-1 in Osteosarcoma Cell MG-63 in an Estrogen Receptor-Independent Manner. *Biochem Biophys Res Commun* (2015) 457(4):500–6. doi: 10.1016/j.bbrc.2014.12.114
371. Hu Q, Li S, Chen C, Zhu M, Chen Y, Zhao Z. 17betaestradiol Treatment Drives Sp1 to Upregulate MALAT1 Expression and Epigenetically Affects Physiological Processes in U2OS Cells. *Mol Med Rep* (2017) 15(3):1335–42. doi: 10.3892/mmr.2017.6115
372. Deng R, Zhang J, Chen J. lncRNA SNHG1 Negatively Regulates Mirna1013p to Enhance the Expression of ROCK1 and Promote Cell Proliferation, Migration and Invasion in Osteosarcoma. *Int J Mol Med* (2019) 43 (3):1157–66. doi: 10.3892/ijmm.2018.4039
373. Ruan W, Wang P, Feng S, Xue Y, Li Y. Long non-Coding RNA Small Nucleolar RNA Host Gene 12 (SNHG12) Promotes Cell Proliferation and Migration by Upregulating Angiomotin Gene Expression in Human Osteosarcoma Cells. *Tumour Biol* (2016) 37(3):4065–73. doi: 10.1007/s13277-015-4256-7
374. Zhou S, Yu L, Xiong M, Dai G. LncRNA SNHG12 Promotes Tumorigenesis and Metastasis in Osteosarcoma by Upregulating Notch2 by Sponging miR-195-5p. *Biochem Biophys Res Commun* (2018) 495(2):1822–32. doi: 10.1016/j.bbrc.2017.12.047
375. Xu N, Xu J, Zuo Z, Liu Y, Yan F, Han C. Downregulation of lncRNA SNHG12 Reversed IGF1R-Induced Osteosarcoma Metastasis and Proliferation by Targeting miR-195-5p. *Gene* (2020) 726:144145. doi: 10.1016/j.gene.2019.144145
376. Wang Y, Zeng X, Wang N, Zhao W, Zhang X, Teng S, et al. Long Noncoding RNA DANCR, Working as a Competitive Endogenous RNA, Promotes ROCK1-Mediated Proliferation and Metastasis via Decoying of miR-335-5p and miR-1972 in Osteosarcoma. *Mol Cancer* (2018) 17(1):89. doi: 10.1186/s12943-018-0837-6
377. Jiang N, Wang X, Xie X, Liao Y, Liu N, Liu J, et al. lncRNA DANCR Promotes Tumor Progression and Cancer Stemness Features in Osteosarcoma by Upregulating AXL *via* miR-33a-5p Inhibition. *Cancer Lett* (2017) 405:46–55. doi: 10.1016/j.canlet.2017.06.009
378. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are Abundant, Conserved, and Associated With ALU Repeats. *RNA* (2013) 19(2):141–57. doi: 10.1261/rna.035667.112
379. Ebbesen KK, Kjems J, Hansen TB. Circular RNAs: Identification, Biogenesis and Function. *Biochim Biophys Acta* (2016) 1859(1):163–8. doi: 10.1016/j.bbagrm.2015.07.007
380. Wang C, Ren M, Zhao X, Wang A, Wang J. Emerging Roles of Circular RNAs in Osteosarcoma. *Med Sci Monit* (2018) 24:7043–50. doi: 10.12659/MSM.912092
381. Xiao-Long M, Kun-Peng Z, Chun-Lin Z. Circular RNA Circ_HIPK3 is Down-Regulated and Suppresses Cell Proliferation, Migration and Invasion in Osteosarcoma. *J Cancer* (2018) 9(10):1856–62. doi: 10.7150/jca.24619
382. Zheng Q, Bao C, Guo W, Li S, Chen J, Chen B, et al. Circular RNA Profiling Reveals an Abundant Circhipk3 That Regulates Cell Growth by Sponging Multiple miRNAs. *Nat Commun* 7:11215. doi: 10.1038/ncomms11215
383. Li Y, Zheng F, Xiao X, Xie F, Tao D, Huang C, et al. CircHIPK3 Sponges miR-558 to Suppress Heparanase Expression in Bladder Cancer Cells. *EMBO Rep* (2017) 18(9):1646–59. doi: 10.15252/embr.201643581
384. Chen G, Shi Y, Liu M, Sun J. Circhipk3 Regulates Cell Proliferation and Migration by Sponging miR-124 and Regulating AQP3 Expression in Hepatocellular Carcinoma. *Cell Death Dis* (2018) 9(2):175. doi: 10.1038/s41419-017-0204-3
385. Kai D, Yannian L, Yitian C, Dinghao G, Xin Z, Wu J. Circular RNA HIPK3 Promotes Gallbladder Cancer Cell Growth by Sponging microRNA-124. *Biochem Biophys Res Commun* (2018) 503(2):863–9. doi: 10.1016/j.bbrc.2018.06.088
386. Zeng K, Chen X, Xu M, Liu X, Hu X, Xu T, et al. CircHIPK3 Promotes Colorectal Cancer Growth and Metastasis by Sponging miR-7. *Cell Death Dis* (2018) 9(4):417. doi: 10.1038/s41419-018-0454-8
387. Wu Z, Shi W, Jiang C. Overexpressing Circular RNA Hsa_Circ_0002052 Impairs Osteosarcoma Progression *via* Inhibiting Wnt/beta-Catenin Pathway by Regulating miR-1205/APC2 Axis. *Biochem Biophys Res Commun* (2018) 502(4):465–71. doi: 10.1016/j.bbrc.2018.05.184
388. Ren C, Liu J, Zheng B, Yan P, Sun Y, Yue B. The Circular RNA Circ-ITCH Acts as a Tumour Suppressor in Osteosarcoma *via* Regulating miR-22. *Artif Cells Nanomed Biotechnol* (2019) 47(1):3359–67. doi: 10.1080/21691401.2019.1649273
389. de Nigris F, Mancini FP, Schiano C, Infante T, Zullo A, Minucci PB, et al. Osteosarcoma Cells Induce Endothelial Cell Proliferation During Neo-Angiogenesis. *J Cell Physiol* (2013) 228(4):846–52. doi: 10.1002/jcp.24234
390. Yamanegi K, Kawabe M, Futani H, Nishiura H, Yamada N, Kato-Kogoe N, et al. Sodium Valproate, a Histone Deacetylase Inhibitor, Modulates the Vascular Endothelial Growth Inhibitor-Mediated Cell Death in Human Osteosarcoma and Vascular Endothelial Cells. *Int J Oncol* (2015) 46 (5):1994–2002. doi: 10.3892/ijo.2015.2924
391. Vimalraj S, Saravanan S, Raghunandhakumar S, Anuradha D. Melatonin Regulates Tumor Angiogenesis *via* miR-424-5p/VEGFA Signaling Pathway in Osteosarcoma. *Life Sci* (2020) 256:118011. doi: 10.1016/j.lfs.2020.118011
392. Bulut G, Hong SH, Chen K, Beauchamp EM, Rahim S, Kosturko GW, et al. Small Molecule Inhibitors of Ezrin Inhibit the Invasive Phenotype of Osteosarcoma Cells. *Oncogene* (2012) 31(3):269–81. doi: 10.1038/onc.2011.245
393. Sleeman J, Steeg PS. Cancer Metastasis as a Therapeutic Target. *Eur J Cancer* (2010) 46(7):1177–80. doi: 10.1016/j.ejca.2010.02.039
394. Steeg PS. Perspective: The Right Trials. *Nature* (2012) 485(7400):S58–9. doi: 10.1038/485S58a
395. Liang C, Li F, Wang L, Zhang ZK, Wang C, He B, et al. Tumor Cell-Targeted Delivery of CRISPR/Cas9 by Aptamer-Functionalized Lipopolymer for Therapeutic Genome Editing of VEGFA in Osteosarcoma. *Biomaterials* (2017) 147:68–85. doi: 10.1016/j.biomaterials.2017.09.015
396. Chou AJ, Gupta R, Bell MD, Riewe KO, Meyers PA, Gorlick R. Inhaled Lipid Cisplatin (ILC) in the Treatment of Patients With Relapsed/Progressive Osteosarcoma Metastatic to the Lung. *Pediatr Blood Cancer* (2013) 60 (4):580–6. doi: 10.1002/pbc.24438
397. Dow S, Elmslie R, Kurzman I, MacEwen G, Pericle F, Liggitt D. Phase I Study of Liposome-DNA Complexes Encoding the Interleukin-2 Gene in Dogs With Osteosarcoma Lung Metastases. *Hum Gene Ther* (2005) 16(8):937–46. doi: 10.1089/hum.2005.16.937
398. Bruland OS, Hoifodt H, Saeter G, Smeland S, Fodstad O. Hematogenous Micrometastases in Osteosarcoma Patients. *Clin Cancer Res* (2005) 11 (13):4666–73. doi: 10.1158/1078-0432.CCR-05-0165
399. Bruland OS, Hoifodt H, Hall KS, Smeland S, Fodstad O. Bone Marrow Micrometastases Studied by an Immunomagnetic Isolation Procedure in

Extremity Localized non-Metastatic Osteosarcoma Patients. *Cancer Treat Res* (2009) 152:509–15. doi: 10.1007/978-1-4419-0284-9_30
400. Guan G, Lu Y, Zhu X, Liu L, Chen J, Ma Q, et al. CXCR4-Targeted Near-Infrared Imaging Allows Detection of Orthotopic and Metastatic Human Osteosarcoma in a Mouse Model. *Sci Rep* (2015) 5:15244. doi: 10.1038/srep15244
401. Wang L, Li P, Xiao X, Li J, Li J, Yang HH, et al. Generating Lung-Metastatic Osteosarcoma Targeting Aptamers for *In Vivo* and Clinical Tissue Imaging. *Talanta* (2018) 188:66–73. doi: 10.1016/j.talanta.2018.05.011

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Oncology

REVIEW
published: 08 July 2022
doi: 10.3389/fonc.2022.946022

Application of Multi-Omics Approach in Sarcomas: A Tool for Studying Mechanism, Biomarkers, and Therapeutic Targets

Zijian Zou[1,2†], *Wei Sun*[1,2†], *Yu Xu*[1,2], *Wanlin Liu*[1,2], *Jingqin Zhong*[1,2], *Xinyi Lin*[1,2] *and Yong Chen*[1,2*]

[1] Department of Musculoskeletal Surgery, Fudan University Shanghai Cancer Center, Shanghai, China,
[2] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China

OPEN ACCESS

Edited by:
Jilong Yang,
Tianjin Medical University Cancer Institute and Hospital, China

Reviewed by:
Haotian Liu,
Tianjin Medical University Cancer Institute and Hospital, China
Chunpu Li,
Tai'an City Central Hospital, China

***Correspondence:**
Yong Chen
chenyong@fudan.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: 17 May 2022
Accepted: 16 June 2022
Published: 08 July 2022

Citation:
Zou Z, Sun W, Xu Y, Liu W, Zhong J, Lin X and Chen Y (2022) Application of Multi-Omics Approach in Sarcomas: A Tool for Studying Mechanism, Biomarkers, and Therapeutic Targets. Front. Oncol. 12:946022. doi: 10.3389/fonc.2022.946022

Sarcomas are rare, heterogeneous mesenchymal neoplasms with various subtypes, each exhibiting unique genetic characteristics. Although studies have been conducted to improve the treatment for sarcomas, the specific development from normal somatic cells to sarcoma cells is still unclear and needs further research. The diagnosis of sarcomas depends heavily on the pathological examination, which is yet a difficult work and requires expert analysis. Advanced treatment like precise medicine optimizes the efficacy of treatment and the prognosis of sarcoma patients, yet, in sarcomas, more studies should be done to put such methods in clinical practice. The revolution of advanced technology has pushed the multi-omics approach to the front, and more could be learnt in sarcomas with such methods. Multi-omics combines the character of each omics techniques, analyzes the mechanism of tumor cells from different levels, which makes up for the shortage of single-omics, and gives us an integrated picture of bioactivities inside tumor cells. Multi-omics research of sarcomas has reached appreciable progress in recent years, leading to a better understanding of the mutation, proliferation, and metastasis of sarcomas. With the help of multi-omics approach, novel biomarkers were found, with promising effects in improving the process of diagnosis, prognosis anticipation, and treatment decision. By analyzing large amounts of biological features, subtype clustering could be done in a better precision, which may be useful in the clinical procedure. In this review, we summarized recent discoveries using multi-omics approach in sarcomas, discussed their merits and challenges, and concluded with future perspectives of the sarcoma research.

Keywords: sarcoma, genomics, transcriptomics, proteomics, metabolomics

INTRODUCTION

Sarcomas are rare malignancies with various subtypes, including more than 70 subtypes of soft tissue sarcomas (STS) and bone sarcomas (1). They are mesenchymal neoplasms with high level of heterogeneity, about 75% of which arise from soft tissue, 15% consist of gastrointestinal stromal tumors, and approximately 10% develop from bones (2). Although STS consists less than 1% of malignant tumor cases in the US (3), the sarcomas account for 15% of the childhood malignancies (4), manifesting the necessity of sarcoma research. The standard treatment for regional STS is surgery. Meanwhile, chemotherapy and radiotherapy serve as neoadjuvant or adjuvant treatment. When it comes to metastasis, chemotherapy may be the appropriate choice for palliative treatment (5). However, current treatments that use the same principle for all sarcoma subtypes have not taken the genetic differences into consideration. Novel therapies like immunotherapy and target therapy gradually come into play, focusing on the discrepancies that define each unique malignancy. Still, most of these precise therapies remain experimental attempts because of the shortage of studies, pointing out the significance of basic analysis for sarcomas.

There are plenty of omics studies of sarcomas, improving our understanding for the mechanism of the sarcomas from different angles. Each type of omics data provides information of the disease, assisting us in finding markers and exploring the biological pathways unique to the disease. For example, prognostic signatures using transcriptomic biomarkers have been established, including Complexity INdex in SARComas (CINSARC) (6), Genomic Grade Index (GGI) (7), and hypoxia-associated signatures (8). Three major situations—the limited effect of loci discovered, the influence from both coding region of genes and gene regulation, and the involvement of environment and genetic background—have made the power of single omics research unable to elucidate the whole picture of the disease development (9). This highlighted the importance of multi-omics approach in sarcoma.

The multi-omics strategies, combining genomics, epigenomics, transcriptomics, proteomics, and metabolomics, is an advanced research method in bioinformatics analysis, which provide us with detailed information for studies and better accuracy. Multi-omics provides a greater understanding of information of one disease, pointing out the direction for the discovery of the cause, the consequence, and the related interaction of the malignancy.

In this review, we provided an overview of multi-omics research for sarcomas in recent years, focusing on their approaches and results, thus discussing the significance of applying multi-omics approaches and providing insights into future perspectives of sarcoma research.

ACHIEVEMENT WITH MULTI-OMICS APPROACH

Mechanism and Diagnosis

Precise diagnosis of sarcoma is not easy, considering its large number of subtypes and relatively small number of cases studied. Multi-omics provide a new aspect to dig deep into the mysterious field, and there have already been various multi-omics studies discovering details of tumorigenesis mechanism that is possibly capable of assisting the diagnosis. The results, the sarcoma subtypes, and the omics techniques applied in each study are summarized in **Table 1**.

Osteosarcoma (OS) lacks a sensitive and specific diagnostic marker, and most patients started their treatment in late stages. Integrating transcriptomic and proteomic data of OS cells, 13 biomarkers were chosen, in which *POLR3F* has most potential to be the diagnostic biomarker for early stages of OS (10). Another OS research integrated transcriptome and metabolome profiles and compared the difference between patients with OS and healthy individuals. Specific genes and metabolites were chosen on the basis of a set of criteria. After the pathway enrichment analysis, the glycolysis/gluconeogenesis pathway was found to be served as the only common pathway enriched in both omics. Finally, four genes (*ENO1*, *TPI1*, *PKG1*, and *LDHC*) and two metabolites (lactate and pyruvate) were chosen to form a signature as the predictive method for OS (11). Rearrangement of *TP53*, *RB1*, and *CDKN2A* was revealed in another study of OS, and a fusion of *PMP22* and *ELOVL5* was newly discovered. The involvement of non-homologous end-joining and microhomology-mediated end-joining DNA repair was confirmed by genomics. The lack of genomic evidence for 17 fusion transcripts, which were detected in the research, suggests a complex mechanism in trans-slicing. Most importantly, it was conformed that *TP53* rearrangement is the leading condition of inactivation of p53 in OS, the frequency of which is much higher in this study than previous statistics (12).

Desmoplastic small round cell tumor (DSRCT) is an aggressive malignancy that occurs mostly in male aged 15 to 35 years, and a *EWSR1-WT1* fusion gene could be found in most cases (27). The upregulation of *WT1* was confirmed in a study using RNA-seq, as well as other neural differentiation–related gene such as *GJB2*, *GAL*, *GALP*, and *ASCL1*. Meanwhile, the SNP arrays showed extensive copy number changes, the most frequent being gain of chromosome arm 1/1q and 5/5p and loss of 6/6q or 16/16q (13). The mechanism underneath needs further exploration.

Early mutations in *TP53* seem to exist in most leiomyosarcomas (LMS) cases, whereas other mutations including *ATRX* deletions and Wnt/β-catenin alternations determine the genetic diversity of LMS (14). In another study, inactivation of *TP53* and *RB1* appears to be a universal phenomenon, together with vast number of copy number variations (CNVs), mutational heterogeneity, and whole-genome duplication. Alternation of telomere maintenance genes like *ATRX*, *RBL2*, and *SP100* is observed as well (15).

A multi-omics study of undifferentiated pleomorphic sarcoma (UPS) of bone revealed recurrent mutations in *TP53* and histone chromatin remodeling genes. Eight somatic fusions were identified, including *CLTC-VMP1* and *FARP1-STK24* that were previously observed in multiple tumors. High expression of *FGF23* was revealed, which may serve as a biomarker of this subtype of sarcoma (16).

TABLE 1 | Major results, sarcoma subtypes, and omics technologies of the multi-omics studies described in this review.

Tumor	Omics	Major Results	Ref.
OS	Transcriptomics and proteomics	*POLR3F* is a potential biomarker of OS.	(10)
OS	Transcriptomics and metabolomics	Four genes (*ENO1*, *TPI1*, *PKG1*, and *LDHC*) and two metabolites (lactate and pyruvate) form a signature of early detection.	(11)
OS	Genomics and transcriptomics	*TP53* rearrangements are the major mechanism of p53 inactivation in osteosarcoma that, with active microhomology-mediated break-induced replication and end-joining repair, contributes to the exceptional chromosomal instability in OS.	(12)
DSRCT	Genomics and transcriptomics	The most common imbalances were gain of chromosomes/chromosome arms 1/1q and 5/5p and loss of 6/6q and 16/16q. *GJB2* and *GAL* that showed higher expression in DSRCT compared with control tumors might be worthwhile to investigate as diagnostic markers at protein level.	(13)
LMS	Genomics and transcriptomics	Early mutations in *TP53* seem to exist in most LMS cases, whereas other mutations including *ATRX* deletions and Wnt/β-catenin alternations determine the genetic diversity of LMS.	(14)
LMS	Genomics and transcriptomics	LMS are characterized by inactivation of *TP53* and *RB1*, with recurrent alterations in telomere maintenance genes such as *ATRX*, *RBL2*, and *SP100*, and commonly display hallmarks of "BRCAness".	(15)
UPSb	Genomics and transcriptomics	Recurrent somatic mutations in *TP53*, recurrent mutations in histone chromatin remodeling genes, including *H3F3A*, *ATRX*, and *DOT1L*, were identified in UPSb. *FGF23* can be a potential molecular biomarker for UPSb.	(16)
MIFS	Genomics and transcriptomics	The most common genomic rearrangements were breakpoints in or around the *OGA*, *NPM3*, and *FGF8* genes in chromosome band 10q24 and loss of 1p11-p21 and 10q26-qter. A breakpoint in or near *TGFBR3* in chromosome 1 was found. Amplification and overexpression of *VGLL3* was a consistent feature in MIFS and MIFS-like tumors.	(17)
DPFT	Genomics and transcriptomics	There is an age-associated differences in the origin of the *COL1A1-PDGFB* fusion and mostly arise after DNA synthesis. Transcriptionally upregulated genes in the amplified regions of chromosomes 17 and 22 were found, including *TBX2*, *PRKCA*, *MSI2*, *SOX9*, *SOX10*, and *PRAME*.	(18)
BCS	Genomics and transcriptomics	There is significant dysregulation of gene expression of epigenetic remodeling agents including key members of the PRC, Sin3A/3b, NuRD, and NcoR/SMRT complexes and the DNA methyltransferases DNMT1, DNMT3a, and DNMT3b.	(19)
OFS	Genomics and transcriptomics	RNASeq confirmed expressed mutations of *DICER1* and *NF1*. Amplification of *MYC* and deletion of *TP53* were found in CNV results.	(20)
UESL	Genomics and transcriptomics	Recurrent breakpoints and overexpression of the chromosome 19 microRNA cluster were observed, together with a *TP53* mutation or copy number loss.	(21)
HS	Genomics and transcriptomics	The PI3K pathway gene *PIK3R6* on chromosome 5 and upregulation of *TNFIAP6* in chromosome 19 were observed strongly associated to histiocytic sarcoma in dogs.	(22)
LMS	Genomics and transcriptomics	The promoter regions of *NPAS4* and *PITX1* genes were selected as the candidate methylation marker loci to distinguish uterine leiomyosarcoma and leiomyoma.	(23)
LPS	Genomics and transcriptomics	Dedifferentiated LPS had higher numbers of somatic copy number losses, amplifications involving Chr 12q, and fusion transcripts than well-differentiated LPS. *HMGA2* and *CPM* rearrangements occur more frequently in dedifferentiated components.	(24)
LPS, LMS	Genomics and transcriptomics	Upregulation of gene expression and gene copy number amplification of *MDM2* and *CDK4* were identified in LMS but not DDLPS. Upregulation of tumor related genes is favored in DDLPS, whereas loss of suppressor function is favored in LMS.	(25)
STS	Genomics and transcriptomics	More frequent *CDKN2A* and *CDKN2B* losses in post-radiation than in sporadic sarcomas. Recurrent *MYC* amplifications and *KDR* variants were detected in post-radiation angiosarcomas.	(26)

OS, osteosarcoma; DSRCT, desmoplastic small round cell tumor; LMS, leiomyosarcoma; UPSb, undifferentiated pleomorphic sarcoma of bone; MIFS, Myxoinflammatory fibroblastic sarcoma; DPFT, dermatofibrosarcoma protuberans family of tumors; BCS, BCOR-CCNB3 sarcoma; OFS, ovarian fibrosarcoma; UESL, undifferentiated embryonal sarcoma of the liver; HS, histiocytic sarcoma; LPS, liposarcoma; STS, soft tissue sarcoma.

Myxoinflammatory fibroblastic sarcoma usually has the feature of translocation t(1;10) (p22-31;q24-25). However, this does not result in an expressed fusion gene; instead, the concomitant hemizygous loss of genes from proximal 1p and transcriptional deregulation of the FGF8 gene in 10q24 may be the reason for the biological process within the cells. In addition, regional loss of distal 10q and 3p and amplification of *VGLL3* showed a high frequency, which may promote the development of this disease (17).

Dermatofibrosarcoma protuberans is a rare sarcoma with a high local recurrent risk and a low metastasis rate. Research on the dermatofibrosarcoma protuberans family of tumors regarded the *COL1A1-PDGFB* fusion with a der(22)t(17;22) or ring chromosome as an age-related feature, and RNASeq identified regions with higher expression, validating former discovery of amplified genes on chromosome 17 and 22. These regions may also serve as therapeutic target point in the future (18).

BCOR-CCNB3 sarcomas, characterized with a BCOR and CCNB3 fusion gene, are round cell undifferentiated sarcomas that share morphologic and immunohistochemical features with Ewing sarcoma (ES). A multi-omics research was conducted for one male patient diagnosed with BCOR-CCNB3 sarcoma of the kidney. The mutation rate was relatively low, and no significant fusion was found besides BCOR-CCNB3. Differentiated gene expression analysis revealed multiple genes encoding key players of the *PRC2*, *PRC4*, *NuRD*, *NCoR*, and *mSIN3A* epi-genetic remodeling complexes, but few DNA methylation changes are correlated to changes in gene expression, suggesting the significant epigenomic dysregulation a consequence rather than a leading cause of tumorigenesis. Numerous changes in miRNA were observed, many of which have known relation with tumorigenesis (19).

Another case study focused on ovarian fibrosarcoma, a rare and aggressive type of sarcoma. The malignancy happened in a 9-year-old girl was diagnosed through morphology. The exome sequencing of both tumor and normal cells of the patient presented a low rate of mutations; when validated by RNASeq,

expressed mutations discovered in previous studies were conformed, which are *DICER1* and *NF1*. Amplification of *MYC* and deletion of *TP53* were found in CNV results, which are commonly discovered in similar neoplasms (20).

Undifferentiated embryonal sarcoma of the liver exhibits recurrent breakpoints affecting the chromosome 19 microRNA cluster. Overexpression of this region was observed in these cases, together with a *TP53* mutation or copy number loss (21).

A multi-omics research on histiocytic sarcoma exhibits a special approach of tumor analysis. The study used flat-coated retrievers that frequently develop a disease of the similar histological and clinical features to histiocytic sarcoma. The PI3K pathway gene *PIK3R6* on canine chromosome 5 was observed strongly associated to histiocytic sarcoma in dogs, and upregulation of *TNFIAP6* in chromosome 19 is considered a risk factor as well (22). The research also shows the potential of dog breeds as excellent subjects for omics analysis, for they have perfect hereditary consistency after years of artificial selection.

Differential diagnosis is crucial for various malignancies, determining the subsequent treatment plans, and multi-omics approach surely casts a new aspect into this field. In an integrated study of genomics, epigenomics, and transcriptomics, Tomoko et al. compared the differences between leiomyoma and LMS in uterine, as a complement to histopathological diagnosis. Extensive chromosomal abnormalities were found in LMS, and eventually the promoter regions of *NPAS4* and *PITX1* genes were selected as the candidate methylation marker loci (23). Dedifferentiated liposarcoma (DDLPS) and well-differentiated liposarcoma are two types of sarcomas, the former frequently progresses to the latter. A study revealed higher copy number loss (CNL), amplification in 12q, and fusion transcripts in DDLPS. There were more rearrangements of *HMGA2* and *CPM* in DDLPS, which may influence the differentiation state of the LPS and need further study to explore (24). Another research focused on the difference between LMS and DDLPS. Through the bioinformatic analysis, amplification of regions encoding *MDM2*, *CDK4*, and *HMGA2* was significant in DDLPS, whereas mutations in *TP53*, *ATRX*, *PTEN*, and *RB1* appeared only in LMS. In addition, the microenvironment analysis showed more endothelial cells and fibroblasts in DDLPS than LMS (25). A comparison between post-radiation and sporadic sarcomas was conducted using genomic and transcriptomic strategies. Higher frequency of *CDKN2A* and *CDKN2B* was found in post-radiation sarcomas than the sporadic ones. Moreover, a high frequency of *MYC* amplification and 8% of *KDR* variants was found in accordance with previous research (26).

Subtypes Clustering

Considering the vast amount of genomic data for malignancies, there are plenty of genetic differences among patients bearing the same type of sarcoma, sometimes making it possible for us to divide them into different subtypes with their respective clinical features and prognosis. Research showed that LMS subtype appeared in an early period of tumor and retained thereafter (14), enhancing the importance of subtype differentiation in malignancies of all stages.

Chondrosarcoma (CS) was divided into six molecular subtypes in a multi-omics research. Integrating DNA, mRNA, and microRNA data, three molecular features were found to influence the clinical outcome: a high mitotic state, regional 14q32 loss of expression, and IDH mutations, leading to genome-wide DNA methylation (28).

LMS are tumors with heterogeneous types, resulting in various prognosis. An analysis of 70 genomes and 130 transcriptomes of LMS observed three expression subtypes. Alternation of muscle related genes, including *DMD* and *MYOCD*, behaved differently in three subtypes, and deletions of the dystrophin gene may suggest an inferior outcome. Importantly, LMS molecular subtyping can be used to reveal its originating tissue, which may help adjust the treatment (14).

Rhabdomyosarcoma (RMS) was divided into two subtypes based on the existence of *PAX3/7* gene fusion. Mutation rates of somatic genes were relatively low in these tumors, especially for those with a *PAX3/7* fusion. As for the fusion negative ones, *RAS* mutation was activated in a large proportion, whereas, in a small part of them, the *BCOR* mutation was newly found (29). The subtypes were divided more precisely in another study. A1 and A2 subtypes, with alveolar histology and *PAX3/7* fusion, presented a worse prognosis. E1 and E2 has frequent CNVs and FGFR4/RAS/AKT pathway mutations and *PTEN* mutations/methylation, whereas E2 processes an extra p53 inactivation and a worse prognosis than E1 (30).

In a comprehensive analysis of sarcomas using RNASeq, CNV, and methylation data, four molecular subtypes (iC1, iC2, iC3, and iC4) with different clinical outcomes were identified, in which iC2 has the worst prognosis and iC4 has an enhanced immune state (31). Another research using The Cancer Genome Atlas (TCGA) database focused on six major types of sarcomas: DDLPS, LMS, UPS, myxofibrosarcoma (MFS), malignant peripheral nerve sheath tumor, and synovial sarcoma. The analysis defined three subtypes of DDLPS, uterine LMS, two subtypes of soft tissue LMS, and merged UPS and MFS as one subtype, whereas others have insufficient samples for further clustering. In addition, the pan-sarcoma analysis of omics data revealed frequent copy number alternations but few somatic mutations (*TP53*, *ATRX*, and *RB1*) in sarcoma, compared with other malignancies. The immune microenvironment analysis suggested its differential influence on different types of sarcomas (32).

Prognostic Biomarkers

Prognosis is a vital variable in patients bearing the same type of sarcoma, yet we lack the powerful tool to anticipate the result. Multi-omics strategies unravel the inner differences between sarcoma cases, thus could partially reveal the possible outcome of them.

OS occur mostly in children and adolescents, which makes it important to identify the prognosis. Up to 25% of the OS cases resulted in metastasis, with pulmonary system being the major site (6). For patients with OS that had lung metastasis, molecules in MAPK signaling pathway and in the PI3K regulatory pathway were abnormally enriched (33). The urokinase plasminogen activator (uPA) is a serine proteinase known to involve in the metastasis of numerous solid tumors. The relationship between lung metastasis and uPA was studied in an integrated analysis

using transcriptomic, proteomic, and secretomic analysis. The uPA/uPAR system regulated through autocrine and paracrine paths was found to be associated with metastatic behavior in OS and could activate other signaling pathways such as MAPK, which also promote the tumor metastasis. This makes activation of the uPA/uPAR axis a potential diagnostic marker for the conversion of OS cells from a non-metastatic to a metastatic phenotype, meanwhile indicating potential drug target for the prevention of metastasis (34). Another research of OS analyzed the genomic and transcriptomic information in cases with different overall survival. Integration of variation and survival data revealed six genes (*MYC*, *CHIC2*, *CCDC152*, *LYL1*, *GPR142*, and *MMP27*) to be the candidates for risk anticipation, and a risk score formulation was established to quantitively calculate the prognosis (35).

STS appears in various genetic forms, yet similarity could still be found in malignancies with unappreciable outcomes, which may serve as prognostic markers. Ribonucleotide reductase subunit M2 (RRM2) highly expresses in STS, and patients who possessed increased RRM2 level had worse overall survival. Overexpression of RRM2 induces proliferation, migration, invasion, and colony formation, whereas silencing of RRM2 arrests the cell cycle at G0/G1 phase and induces apoptosis (36). The result suggests that RRM2 could be a potential prognostic marker and a promising therapeutic target in STS. In another research, CNV of RNA regulatory genes were widely observed in STS patients, and there are possible relations between CNV and overall survival of patients. CNL of *METTL4* was associated with worse overall survival in both LMS and DDLPS. A low infiltration fraction of resting mast cells, which was found related to poor overall survival, was discovered in patient with LMS with CNL of *METTL4*. Facts above define CNL of *METTL4* as a potential prognostic marker for STS (37).

UPS is a subtype of STS with a commonly poor outcome, yet no biomarker is used at present to estimate the prognosis. Using two sets of transcriptomic data, high mRNA level of adenosine monophosphate deaminase 2 (AMPD2) was found correlated to a worse outcome. After comparing to CNV information, it was confirmed that CNVs promote AMPD2 expression in UPS. Together with the finding that AMPD2 may promote tumor growth and proliferation, AMPD2 could be a promising prognostic biomarker for UPS (38).

The expression level of replication-dependent histone mRNAs rises in DDLPS, compared with normal tissues, which is the result of the overexpression of *HMGA2*. The correlation between replication-dependent histone level and the aggressiveness of tumor makes it a promising prognosis marker (39).

E-cadherin protein expression was detected in LMS in a multi-omics study, which is also related to a better survival. The inverse correlation between E-cadherin repressor Slug expression and E-cadherin was also revealed, and in conclusion, the mesenchymal-to-epithelial reverting transition regulated by Slug appears to be a significant phenotype of LMS (40).

In a study of sarcomas, seven proteins related to overall survival (AMPKALPHA, CHK1, S6, ARID1A, RBM15, ACETYLATUBULINLYS40, and MSH6) were discovered using the proteomics data of different sarcoma subtypes. A prognostic risk signature was established and validated through transcriptomic profile (41). *ENO1*, *ACVRL1*, and *APBB1IP* were determined to be the prognostic biomarkers for sarcomas in another research, after comparing the four molecular subtypes divided on the basis of prognostic data (31). Another study used transcriptomic data to estimate the level of tumor-infiltrating lymphocytes. It was found that the infiltration of $CD4^+$ T cell, influenced mainly by the CNVs, is related to the overall survival of sarcoma patients, especially for UPS (42).

Treatment Evaluation

Currently, patients with sarcoma would receive surgical resection, radiotherapy, or systemic chemotherapy treatment as the standard routine. However, these may not be enough for patients with metastasis and recurrent situations. In fact, the 5-year survival rates of OS have not seen significant improvement in the past three decades (43). The ultimate purpose of medical research is to prevent and cure the existing disease, so the evaluation of drug efficacy is necessary. The omics approach focuses on the molecular level and may find the novel therapeutic biomarkers, especially for the targeted drugs.

CNV of *METTL4* in STS has a correlation with IC50 of 12 drugs including Temozolomide and Olaparib, suggesting a lower concentration need in clinical practice (37). Homologous recombination deficiency signature appeared in a large proportion of the LMS cases, as well as in other sarcomas, which may serve as a therapeutic biomarker, indicating the possibility of introducing PARP inhibitors (14, 15, 24, 44). An integrated study of DDLPS combined genomics, epigenomics, and transcriptomics data of four samples discovered a high frequency of *CEBPA* methylation. The methylation and silencing of miR-193b was found in patients bearing aggressive disease, suggesting its importance in liposarcoma genesis. Application of demethylating agents had an anti-proliferative and pro-apoptotic effect both *in vitro* and *in vivo* in the experiment, indicating a potential therapeutic method (45). Common receptor of tyrosine kinase/*RAS*/*PIK3CA* genetic axis was found in RMS tumors, which may be an option for targeted therapy (29). A study of DSRCT showed a low rate of somatic mutation but a large number of CNVs. Amplification of *FGFR4*, which is highly expressed in DSRCT cells, suggests a therapeutic potential and needs further study for a better comprehension of the mechanism within (46). Epithelioid sarcoma presented a high mutation rate and complex genomic characteristics. *SMARCB1* was the most frequent mutation, the loss of which leads to the aberrations in *SWI/SNF*, indicating its potential role in the abundance of translocations in epithelioid sarcoma. Because the *SWI/SNF* complex affects the proliferation of the tumor *in vitro*, there may be possibility for novel treatment (47).

Ginsenoside Rh2, a ginseng extract inhibiting the proliferation, migration, and invasion in multiple tumors, has the effect of anti-proliferation and apoptosis-induction in metastatic OS cells and inhibits migration by reversing epithelial-to-mesenchymal transition and promoting degradation of extracellular matrix (33). TRAF2- and NCK-interacting protein kinase (TNIK) was previously recognized as

an essential factor of Wnt signaling pathway, whose inhibition leads to the loss of cell stemness. In a recent study, the suppression of OS cells was observed after applying TNIK inhibitor. Through transcriptomic analysis, pathways related to metabolism were upregulated, whereas pathways involved in Wnt signaling and pathways regulating the pluripotency of stem cells were downregulated, indicating the abrogation of OS stemness. Further metabolomics analysis discovered that drug-induced OS cells favored oxidative phosphorylation, unlike normal tumor cells that present aerobic glycolysis in a great extent (48). The result supports the potential of TNIK as a biomarker for molecular treatment, considering the fact that no current targeted therapy is approved for OS (49).

Immunotherapy shows promising effects in multiple tumors, but its attempts in sarcoma have not been desirable. Nine immune checkpoint-related lncRNAs (CD274, CD80, CD86, PDCD1 LG2, and LGALS9) were identified in a multi-omics research, the overexpression of which suggesting a worse outcome. Further investigation revealed their function of negative regulation in immune response, indicating a possible cause for the immunosuppressive environment in sarcomas, and may be a breakpoint in immunotherapy (50).

Metastasis is a common consequence of sarcomas, and a multi-omics study focused on the biological character of the metastatic sarcomas, trying to find a new therapeutic option. Structural variation and CNVs appeared to be the most frequent changes, and recurrent 17p11-12 amplifications were observed, suggesting a potential point for further research and treatment discovery (44).

To find drug efficacy biomarkers for childhood sarcomas, Brian et al. established tumor xenograft models representing most childhood malignancies. Models were tested using standard anti-tumor agents (vincristine and eribulin) and then studied with novel drugs (alisertib, volasertib, glembatumumab vedotin, and NTX-010). CNV and mRNA data of sensitive models were analyzed, and potential sensitivity biomarkers were discovered in most cases (51). In addition, these models are capable of further omics research in the future.

ADVANTAGES AND DRAWBACKS OF APPLYING MULTI-OMICS APPROACH

With the development of technology, precise and convenient research methods continue to alter the process of analysis, giving us insights into the depth of science. The omics approach collects mass quantity of data, analyzes the data with various mathematical methods, and finally recognizes abnormal biological features, the routine of which require much effort and time in early days, yet modern analysis software and advanced statistic formulas have made it easy even for analysis of multi-omics.

Multi-omics approach applies vast amount of data, compared with traditional sarcoma research. The integration of different types of omics data, combined with prior knowledge of regulation pathways, shows us a much more precise vision of activities inside sarcoma cells and may discover novel regulatory mechanisms as well. Unsupervised integrations identify every correlation among omics data, extracting biological process behind cancer progression, whereas supervised methods based on known phenotype knowledge improve the accuracy for cancer detection (52). These advanced research methods allow us to further study the complete picture of sarcoma as well as other malignancies, with accuracy, reliability, and comprehensiveness.

Another bonus point for multi-omics approach is the accessibility of data on the internet. The omics data take in almost the whole information of one aspect of a sample, which means great potential in mechanism discovery and feasibility for multiple analysis using different methods. On the basis of this idea, plenty of websites containing omics data had already been established. For example, TCGA, a database containing information of more than 30 human tumor types, catalogs aberrations in the DNA and chromatin of the cancer-genomes from thousands of tumors (53). There are other databases like Gene Expression Omnibus (GEO), Expression Atlas, and Oncomine, providing data in different aspects. Because of the low incidence rate of sarcomas, it is hard to obtain enough omics data for an integrated analysis, and such database provides an opportunity to gather data from all over the world so that a proper multi-omics research could be conducted. In fact, many of the studies above used information from online databases, as a supplement to their data of sarcoma cases. In addition, advanced technology has decreased the analytic burden with various computational algorithms like iCluster (54) and iOmicsPASS (55). Functional enrichment tools help cluster the variations through biological function, and online database equipped with these tools like Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) has made it easier for researchers to obtain the result. Because there are only few cases of sarcomas to be studied, the multi-omics approach provides a relatively efficient way to collect as much information as possible.

Although the advantages of multi-omics sarcoma research introduce convenience, there are deficiencies that need further improvement. The extremely low incidence rate of sarcoma brings obstacles for collecting abundant samples, not to mention their omics data. As a result, many of the multi-omics studies above used data from online databases, with little newly sequenced data from local patients, which may lead to bias because of the recurrent utilization of same sets of data. For those that only use samples from local patients, some of them were even not able to reach the standard of biomarker distinguishing because of the improper size of data. To obtain results with more stability and reliability, larger sets of omics data of sarcomas are needed. Another vital problem comes from the constitution of sarcomas. Although the therapeutic principle is "one-size-fits-all" in recent treatments, the inevitable fact that sarcomas variate in numerous subtypes should be taken into consideration when introducing precise medicine. As another result of insufficient data, several present studies roughly put all STS subtypes in one analysis, regardless of their genetic differences.

The expense of research should be discussed as well. Obviously, the omics study obtains much more information from patients' tissue than traditional methods, despite the rise

of cost. However, the omics approach did not come into stage with accuracy only. Technological advances, like expression quantitative trait loci, had made it possible for cost-efficient, high-throughput analysis of molecules (5).

The establishment of omics databases promotes the development of multi-omics study in a large extent, yet there are problems need to be fixed as well. Several databases containing information of different sources provide an opportunity for analysis of large number of samples, except for one question: the lack of a statistical standard. For example, in GEO database, there are data of progression free survival for each patient with UPS, whereas only time of death could be found in TCGA (41). Blindly combining data of distinct statistical standards could lead to conclusions with vital error; therefore, the universal standard for omics data collection is in urgent need. In addition to lack of a statistical standard, batch effects exist between different databases and sequencing data, which is also a problem to be solved in omics research.

FUTURE PERSPECTIVES AND CONCLUSIONS

The use of multi-omics in sarcoma research has just started, and there are methods and process that could be improved, but the future application of multi-omics approach indeed has promising value. The systematic analysis of omics data can characterize the intersection of different level of information, decipher the molecular mechanism in tumor occurrence and development, and possibly anticipate the evolutionary process of specific cells. The sarcomas, many subtypes of which have complex karyotypes (32), are suitable for such analysis to explore the underlying characteristics. With the advanced analytical methods, molecular modeling algorithms, and computational tools, more could be discovered in diagnosis, prognosis, and treatment guidelines.

As we discussed above, the lack of sarcoma cases makes it difficult to conduct a proper analysis with enough data. Studies with larger sample sizes are required, which was mentioned by many of the multi-omics analysis of sarcoma. However, because of the low incidence rate of sarcomas, it is merely possible for one institute to gather enough sarcoma samples with sufficient omics data. The online database with a universal statistical standard may be one solution for this problem. The omics data, each representing the whole biological information of one level of cancer cells, contain abundant knowledge that could be repeatedly explored using different methods, which makes it valuable material that worth reserving for future research. Assembling information from several institutes may establish an aggregation capable of analysis for different types of sarcomas and, with the continuous update of analytic strategies and persistent expansion of data size, frequently reach novel discoveries.

Among the multi-omics studies on sarcoma, genomics, epigenomics, transcriptomics, and proteomics were widely applied on the basis of their respective demands, but few of the multi-omics research had considered metabolomics until recently (**Figure 1**). One reason for this phenomenon is the relative sparseness in knowledge of sarcoma metabolome, which act as an obstacle for the metabolomics study. Another reason may be the fact that the exploration of metabolomics of all tumors is still on the beginning point at present. Tumor cells survive in a reorganized, stressful, and metabolically competitive environment, forcing them to adapt to available metabolites for the reservation of cell plasticity (56). The metabolomics, evaluating metabolites in biospecimen for a better understanding of the full view of metabolism in malignancies, may lead to the discovery of new biomarkers and targets for advanced treatment. Sarcomas, displaying abnormal metabolic activity patterns, still need much efforts of exploration to link these situations to specific gene mutations (57). Common step of metabolomics analysis includes sample collection and preparation, metabolite extraction, data acquisition, analysis, and interpretation, and there are already results for OS in metabolic biomarkers of diagnosis, chemoresistance, and metastasis (58). When integrated with other omics data, there would be a better understanding of the metabolic pathways from the gene level.

In sarcoma research above, plenty characteristics of different omics were discovered (**Table 2**). Several types of sarcomas exhibit relatively low rate of somatic mutation, and such phenomenon was observed in some of the multi-omics studies, whereas a large number of CNVs were discovered, along with structural mutations and DNA methylation (13, 19, 20, 23, 29, 32, 37, 42, 46). The few tumorigenic driver genes, together with the genetic amplifications and deletions, construct an environment suitable for the development of malignancy. In addition, processes like trans-slicing altered the protein expression, contributing to the complexity of sarcoma cells. Such results reveal the shortage of traditional genomic research, which focuses mainly on somatic mutations and exaggerates the dominance of DNA sequence. Multi-omics

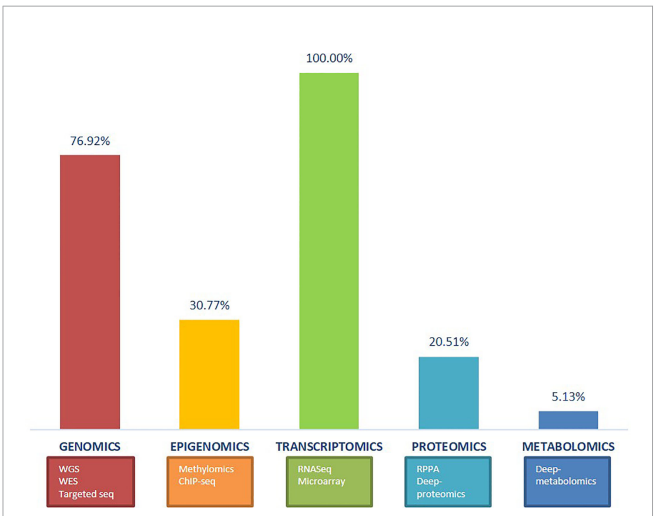


FIGURE 1 | The frequency of use for each omics technologies in the multi-omics studies mentioned in this review. The frames below list the common methods of each omics.

studies on these sarcomas should probably put more attention on the CNVs and DNA methylations and capture their inner relations, which could lead to a more complete picture of sarcomas.

The research process of *TP53* gene fully embodies the value of the application of multi-omics. *TP53* is the gene of a transcription factor that induces gene involved in cell cycle arrest, apoptosis, and metabolism, thus playing an important role in the development of tumors. The majority of *TP53* mutations are missense mutations, which also gains oncogenic functions, leading to a worse outcome (59). The mutation rate of *TP53* was thought to be relatively low in previous studies (60), because only methods like exome sequencing were conducted on sarcoma samples. However, more alternations of *TP53* pathway in sarcomas were found in recent studies (12, 15, 16, 20, 21, 25, 29, 30), including genomic rearrangements, CNVs, expression regulations, and, in some cases, alternation of *TP53*, leading to a worse prognosis. This indicates the nonnegligible effect of *TP53* in sarcomas, and the proportion of *TP53* alternation of all kinds in sarcomas could be larger than previously expected. More should be studied in *TP53* in sarcoma research, and with the help of multi-omics, we could reveal the details of whole *TP53* regulation axis, figure out the influence of alternation in each step, and finally come up with methods to treat, to predict, and to prevent the deterioration of *TP53*-related sarcomas.

Many of the studies used multiple biomarkers to form a prognostic formula, which beats the single-biomarker prognosis considering the complexity of sarcoma tumorigenesis. However, some attempts of personalized treatment using genomic data did not come up with good results, indicating that genomic only is not enough for the precise prediction of therapy response (61). One of the studies uses a signature integrated with gene and metabolite levels (11), which could be a promising aspect of research, especially for multi-omics research. However, the

TABLE 2 | Alternations of omics found in research of major sarcoma subtypes.

Tumor	OS	CS	DSRCT	LMS	RMS	DDLPS	UPS
MicroRNA Alternation		miR-27B, miR-125A, miR-140, miR-154, miR-382, miR-384		miR-181b-5p		miR-193b	miR-100-5p, miR-194-5p
Overexpression	ENO1, TPI1, PKG1, LDHC, SMARCA2, BAZ2A, POLR3F, CYC1, PITPNC1, PDCD2, DKK3, HS2ST1, UCHL3, DNASE2, WDR12, SKIP		FGFR4	ARL4C, CASQ2, LMOD1	PTPN11, ATM, ZNF350, TRPC4AP, FOXO1, ARID1A	MDM2, HMGA2, CDK4, GINS4, BRCA2, XRCC2, RAD51AP1, RAD51, RAD54B, XRCC1, POLQ, FEN1	FGF23
Gene Fusion	PMP22-ELOVL5		EWSR1-WT1	HMGA2-CPM	PAX3/7–FOXO1		APOL1-MYH9, PKNOX2-MMP20, ASAP2-ADAM17, CLTC-VMP1, FARP1-STK24
Structural Variation	TP53, RB1, CDKN2A, MDM2, MTAP			PTEN, BRCA1/2, ATM, FANCA, CHEK1, XRCC3, CHEK2, RAD51, FANCD2	CDKN2A, MIR17HG, CNR1, ERBB4, RPTOR, FRS2, CACNA1A, NRG1, FOXP2		
Methylation				PITX1		CEBPA	
Copy Number Variation	RB1, CDKN2A, CLU, BNIP3, IGFBP3, SPARC, TPD52, MEST, PRG1, OXTR, LOXL2, PTGFR, LYL1, DLG2	CDKN2A	GAL, GALP, ASCL1	TP53, RB1, CDKN2A, PTEN, ATRX, RBL2, BRCA1/2, ATM, FANCA, ALK, FGFR2, LIFR, PAX3, CDX2, SUFU, CDH1, DMD, MYOCD, DNMT3A, KAT6B, FLT3, FOXO1,	TP53, CDKN2A/B, MYCN, MDM2, MET, ALK, FGFR4, STAT6, IGF1R, MIR17HG, FRS2, MYOD1, CNR1	TP53, CDKN2A, MDM2, TERT, HMGA2, CDK4, ATRX, YEATS2, NF1, FRS2, NAV3	RB1, CDKN2A, MDM2, ING1, MYC, PDGFRA, KIT, KDR, PDGFA, PDGFB, VEGFA, CCNE1, YAP1, VGLL3
Mutation		IDH1/2, COL2A	TP53, TERT, ARID1A, HRAS	TP53, RB1, PTEN, PSDM11, CASP7, XPO1, SETD7, MTOR, ATRX, TOPORS, ATR, TP53BP1, TELO2, KMT2C, MAPK14, DUSP10, ZFP36L1, SRSF5, MED12, FH, MEF2C, HIST3H3, LAMA4	TP53, BCOR, CCND2, ARID1A, NRAS, KRAS, HRAS, FGFR4, PIK3CA, CTNNB1, IGF1/2, FBXW7	HERC2, MAPKAP1, HDAC1, DAZAP2, PTPN9	TP53, ATRX, H3F3A, DOT1L
Ref.	(10–12, 35)	(28)	(13, 46)	(14, 15, 23, 25, 32)	(29, 30)	(24, 25, 32, 39, 45)	(16, 32)

OS, osteosarcoma; CS, chondrosarcoma; DSRCT, desmoplastic small round cell tumor; LMS, leiomyosarcoma; RMS, rhabdomyosarcoma; DDLPS, dedifferentiated liposarcoma; UPS, undifferentiated pleomorphic sarcoma.

application of such strategy needs thorough understanding of pathways involved in tumorigenesis, which is yet a relatively undeveloped field for sarcomas. Still, integrated signature could definitely fill the gap caused by differential expression and lead to a prognosis with lower false rate in the future.

Recent advances in high-throughput technologies have enabled the large-scale data analysis in genomics as well as other omics fields, which revolutionize the approaches in medical studies. Sarcoma research with the multi-omics strategies has reached various novel results, yet the vast unexplored field of unsolved issues needs further studies to fill in the blank. The integration of omics data, paving the way to a deeper understanding of the characteristics of sarcomas, may lead us to a more precise diagnosis, a more accurate prognosis, and more potential biomarkers for novel drug treatments.

AUTHOR CONTRIBUTIONS

ZZ and WS wrote and edited this manuscript and created tables and figure. ZZ, WS, YX, WL, JZ, XL, and YC reviewed and revised the manuscript. WS and YC provided direction and guidance throughout the preparation of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by Shanghai Science and Technology Development Funds (19411951700), the Lingang Laboratory (Grant No. LG-QS-202205-11), and the national natural science foundation of China (81802636).

REFERENCES

1. Hui JY. Epidemiology and Etiology of Sarcomas. *Surg Clin North Am* (2016) 96(5):901–14. doi: 10.1016/j.suc.2016.05.005
2. Casali PG, Abecassis N, Aro HT, Bauer S, Biagini R, Bielack S, et al. Soft Tissue and Visceral Sarcomas: ESMO-EURACAN Clinical Practice Guidelines for Diagnosis, Treatment and Follow-Up. *Ann Oncol* (2018) 29 (Suppl 4):iv51–67. doi: 10.1093/annonc/mdy096
3. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. *CA Cancer J Clin* (2021) 71(1):7–33. doi: 10.3322/caac.21654
4. von Mehren M, Kane JM, Bui MM, Choy E, Connelly M, Dry S, et al. NCCN Guidelines Insights: Soft Tissue Sarcoma, Version 1.2021. *J Natl Compr Canc Netw* (2020) 18(12):1604–12. doi: 10.6004/jnccn.2020.0058
5. Bourcier K, Le Cesne A, Tselikas L, Adam J, Mir O, Honore C, et al. Basic Knowledge in Soft Tissue Sarcoma. *Cardiovasc Intervent Radiol* (2019) 42 (9):1255–61. doi: 10.1007/s00270-019-02259-w
6. Chibon F, Lagarde P, Salas S, Pérot G, Brouste V, Tirode F, et al. Validated Prediction of Clinical Outcome in Sarcomas and Multiple Types of Cancer on the Basis of a Gene Expression Signature Related to Genome Complexity. *Nat Med* (2010) 16(7):781–7. doi: 10.1038/nm.2174
7. Bertucci F, Finetti P, Ostrowski J, Kim WK, Kim H, Pantaleo MA, et al. Genomic Grade Index Predicts Postoperative Clinical Outcome of GIST. *Br J Cancer* (2012) 107(8):1433–41. doi: 10.1038/bjc.2012.390
8. Yang L, Forker L, Irlam JJ, Pillay N, Choudhury A, West CML. Validation of a Hypoxia Related Gene Signature in Multiple Soft Tissue Sarcoma Cohorts. *Oncotarget* (2018) 9(3):3946–55. doi: 10.18632/oncotarget.23280
9. Hasin Y, Seldin M, Lusis A. Multi-Omics Approaches to Disease. *Genome Biol* (2017) 18(1):83. doi: 10.1186/s13059-017-1215-1
10. Li GD, Cai ZD, Ma XJ, Sun MX, Li J. [Application of an Integrated Omics Analysis for the Discovery of Biomarkers for Osteosarcoma]. *Zhonghua Yi Xue Za Zhi* (2012) 92(3):192–6. doi: 10.3760/cma.j.issn.0376-2491.2012.03.012
11. Chen K, Zhu C, Cai M, Fu D, Cheng B, Cai Z, et al. Integrative Metabolome and Transcriptome Profiling Reveals Discordant Glycolysis Process Between Osteosarcoma and Normal Osteoblastic Cells. *J Cancer Res Clin Oncol* (2014) 140(10):1715–21. doi: 10.1007/s00432-014-1719-y
12. Lorenz S, Barøy T, Sun J, Nome T, Vodák D, Bryne JC, et al. Unscrambling the Genomic Chaos of Osteosarcoma Reveals Extensive Transcript Fusion, Recurrent Rearrangements and Frequent Novel TP53 Aberrations. *Oncotarget* (2016) 7(5):5273–88. doi: 10.18632/oncotarget.6567
13. Sydow S, Versleijen-Jonkers YMH, Hansson M, van Erp AEM, Hillebrandt-Roeffen MHS, van der Graaf WTA, et al. Genomic and Transcriptomic Characterization of Desmoplastic Small Round Cell Tumors. *Genes Chromosomes Cancer* (2021) 60(9):595–603. doi: 10.1002/gcc.22955
14. Anderson ND, Babichev Y, Fuligni F, Comitani F, Layeghifard M, Venier RE, et al. Lineage-Defined Leiomyosarcoma Subtypes Emerge Years Before Diagnosis and Determine Patient Survival. *Nat Commun* (2021) 12(1):4496. doi: 10.1038/s41467-021-24677-6
15. Chudasama P, Mughal SS, Sanders MA, Hübschmann D, Chung I, Deeg KI, et al. Integrative Genomic and Transcriptomic Analysis of Leiomyosarcoma. *Nat Commun* (2018) 9(1):144. doi: 10.1038/s41467-017-02602-0
16. Ali NM, Niada S, Brini AT, Morris MR, Kurusamy S, Alholle A, et al. Genomic and Transcriptomic Characterisation of Undifferentiated Pleomorphic Sarcoma of Bone. *J Pathol* (2019) 247(2):166–76. doi: 10.1002/path.5176
17. Arbajian E, Hofvander J, Magnusson L, Mertens F. Deep Sequencing of Myxoinflammatory Fibroblastic Sarcoma. *Genes Chromosomes Cancer* (2020) 59(5):309–17. doi: 10.1002/gcc.22832
18. Köster J, Arbajian E, Viklund B, Isaksson A, Hofvander J, Haglund F, et al. Genomic and Transcriptomic Features of Dermatofibrosarcoma Protuberans: Unusual Chromosomal Origin of the COL1A1-PDGFB Fusion Gene and Synergistic Effects of Amplified Regions in Tumor Development. *Cancer Genet* (2020) 241:34–41. doi: 10.1016/j.cancergen.2019.12.001
19. Hagoel TJ, Cortes Gomez E, Gupta A, Twist CJ, Kozielski R, Martin JC, et al. Clinicopathologic and Molecular Analysis of a BCOR-CCNB3 Undifferentiated Sarcoma of the Kidney Reveals Significant Epigenetic Alterations. *Cold Spring Harb Mol Case Stud* (2022) 8(1):a005942. doi: 10.1101/mcs.a005942
20. Melendez-Zajgla J, Mercado-Celis GE, Gaytan-Cervantes J, Torres A, Gabiño NB, Zapata-Tarres M, et al. Genomics of a Pediatric Ovarian Fibrosarcoma. Association With the DICER1 Syndrome. *Sci Rep* (2018) 8(1):3252. doi: 10.1038/s41598-018-21663-9
21. Setty BA, Jinesh GG, Arnold M, Pettersson F, Cheng CH, Cen L, et al. The Genomic Landscape of Undifferentiated Embryonal Sarcoma of the Liver is Typified by C19MC Structural Rearrangement and Overexpression Combined With TP53 Mutation or Loss. *PloS Genet* (2020) 16(4):e1008642. doi: 10.1371/journal.pgen.1008642
22. Evans JM, Parker HG, Rutteman GR, Plassais J, Grinwis GCM, Harris AC, et al. Multi-Omics Approach Identifies Germline Regulatory Variants Associated With Hematopoietic Malignancies in Retriever Dog Breeds. *PloS Genet* (2021) 17(5):e1009543. doi: 10.1371/journal.pgen.1009543
23. Miyata T, Sonoda K, Tomikawa J, Tayama C, Okamura K, Maehara K, et al. Genomic, Epigenomic, and Transcriptomic Profiling Towards Identifying Omics Features and Specific Biomarkers That Distinguish Uterine Leiomyosarcoma and Leiomyoma at Molecular Levels. *Sarcoma* (2015) 2015:412068. doi: 10.1155/2015/412068
24. Beird HC, Wu CC, Ingram DR, Wang WL, Alimohamed A, Gumbs C, et al. Genomic Profiling of Dedifferentiated Liposarcoma Compared to Matched Well-Differentiated Liposarcoma Reveals Higher Genomic Complexity and a Common Origin. *Cold Spring Harb Mol Case Stud* (2018) 4(2):a002386. doi: 10.1101/mcs.a002386
25. Liu W, Tong H, Zhang C, Zhuang R, Guo H, Lv C, et al. Integrated Genomic and Transcriptomic Analysis Revealed Mutation Patterns of De-Differentiated

Liposarcoma and Leiomyosarcoma. *BMC Cancer* (2020) 20(1):1035. doi: 10.1186/s12885-020-07456-2
26. Lesluyes T, Baud J, Pérot G, Charon-Barra C, You A, Valo I, et al. Genomic and Transcriptomic Comparison of Post-Radiation Versus Sporadic Sarcomas. *Mod Pathol* (2019) 32(12):1786–94. doi: 10.1038/s41379-019-0300-2
27. Kang HJ, Park JH, Chen W, Kang SI, Moroz K, Ladanyi M, et al. EWS-WT1 Oncoprotein Activates Neuronal Reprogramming Factor ASCL1 and Promotes Neural Differentiation. *Cancer Res* (2014) 74(16):4526–35. doi: 10.1158/0008-5472.CAN-13-3663
28. Nicolle R, Ayadi M, Gomez-Brouchet A, Armenoult L, Banneau G, Elarouci N, et al. Integrated Molecular Characterization of Chondrosarcoma Reveals Critical Determinants of Disease Progression. *Nat Commun* (2019) 10 (1):4622. doi: 10.1038/s41467-019-12525-7
29. Shern JF, Chen L, Chmielecki J, Wei JS, Patidar R, Rosenberg M, et al. Comprehensive Genomic Analysis of Rhabdomyosarcoma Reveals a Landscape of Alterations Affecting a Common Genetic Axis in Fusion-Positive and Fusion-Negative Tumors. *Cancer Discovery* (2014) 4(2):216–31. doi: 10.1158/2159-8290.CD-13-0639
30. Seki M, Nishimura R, Yoshida K, Shimamura T, Shiraishi Y, Sato Y, et al. Integrated Genetic and Epigenetic Analysis Defines Novel Molecular Subgroups in Rhabdomyosarcoma. *Nat Commun* (2015) 6:7557. doi: 10.1038/ncomms8557
31. Song Y, Yang K, Sun T, Tang R. Development and Validation of Prognostic Markers in Sarcomas Base on a Multi-Omics Analysis. *BMC Med Genomics* (2021) 14(1):31. doi: 10.1186/s12920-021-00876-4
32. Cancer Genome Atlas Research Network, Electronic address edsc and Cancer Genome Atlas Research N. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. *Cell* (2017) 171(4):950–65.e28. doi: 10.1016/j.cell.2017.10.014
33. Liu D, Wang H, Zhou Z, Mao X, Ye Z, Zhang Z, et al. Integrated Bioinformatic Analysis and Experiment Confirmation of the Antagonistic Effect and Molecular Mechanism of Ginsenoside Rh2 in Metastatic Osteosarcoma. *J Pharm BioMed Anal* (2021) 201:114088. doi: 10.1016/j.jpba.2021.114088
34. Endo-Munoz L, Cai N, Cumming A, Macklin R, Merida de Long L, Topkas E, et al. Progression of Osteosarcoma From a Non-Metastatic to a Metastatic Phenotype Is Causally Associated With Activation of an Autocrine and Paracrine uPA Axis. *PloS One* (2015) 10(8):e0133592. doi: 10.1371/journal.pone.0133592
35. Li R, Wang G, Wu Z, Lu H, Li G, Sun Q, et al. Identification of 6 Gene Markers for Survival Prediction in Osteosarcoma Cases Based on Multi-Omics Analysis. *Exp Biol Med (Maywood)* (2021) 246(13):1512–23. doi: 10.1177/1535370221992015
36. Das B, Jain N, Mallick B. Ribonucleotide Reductase Subunit M2 is a Potential Prognostic Marker and Therapeutic Target for Soft Tissue Sarcoma. *Gene* (2022) 808:145988. doi: 10.1016/j.gene.2021.145988
37. Wang X, Han Y, Li J, Hong D, Xue Z, Huang H, et al. Multi-Omics Analysis of Copy Number Variations of RNA Regulatory Genes in Soft Tissue Sarcoma. *Life Sci* (2021) 265:118734. doi: 10.1016/j.lfs.2020.118734
38. Orth MF, Gerke JS, Knösel T, Altendorf-Hofmann A, Musa J, Alba-Rubio R, et al. Functional Genomics Identifies AMPD2 as a New Prognostic Marker for Undifferentiated Pleomorphic Sarcoma. *Int J Cancer* (2019) 144(4):859–67. doi: 10.1002/ijc.31903
39. Yoo Y, Park SY, Jo EB, Choi M, Lee KW, Hong D, et al. Overexpression of Replication-Dependent Histone Signifies a Subset of Dedifferentiated Liposarcoma With Increased Aggressiveness. *Cancers (Basel)* (2021) 13 (13):3122. doi: 10.3390/cancers13133122
40. Yang J, Eddy JA, Pan Y, Hategan A, Tabus I, Wang Y, et al. Integrated Proteomics and Genomics Analysis Reveals a Novel Mesenchymal to Epithelial Reverting Transition in Leiomyosarcoma Through Regulation of Slug. *Mol Cell Proteomics* (2010) 9(11):2405–13. doi: 10.1074/mcp.M110.000240
41. Zhang B, Yang L, Wang X, Fu D. Identification of a Survival-Related Signature for Sarcoma Patients Through Integrated Transcriptomic and Proteomic Profiling Analyses. *Gene* (2021) 764:145105. doi: 10.1016/j.gene.2020.145105
42. Bi Q, Liu Y, Yuan T, Wang H, Li B, Jiang Y, et al. Predicted CD4(+) T Cell Infiltration Levels Could Indicate Better Overall Survival in Sarcoma Patients. *J Int Med Res* (2021) 49(1):300060520981539. doi: 10.1177/0300060520981539
43. Nie Z, Peng H. Osteosarcoma in Patients Below 25 Years of Age: An Observational Study of Incidence, Metastasis, Treatment and Outcomes. *Oncol Lett* (2018) 16(5):6502–14. doi: 10.3892/ol.2018.9453
44. Feng X, Pleasance E, Zhao EY, Ng T, Grewal JK, Mohammad N, et al. Therapeutic Implication of Genomic Landscape of Adult Metastatic Sarcoma. *JCO Precis Oncol* (2019) 3:1–25. doi: 10.1200/PO.18.00325
45. Taylor BS, DeCarolis PL, Angeles CV, Brenet F, Schultz N, Antonescu CR, et al. Frequent Alterations and Epigenetic Silencing of Differentiation Pathway Genes in Structurally Rearranged Liposarcomas. *Cancer Discovery* (2011) 1 (7):587–97. doi: 10.1158/2159-8290.CD-11-0181
46. Slotkin EK, Bowman AS, Levine MF, Dela Cruz F, Coutinho DF, Sanchez GI, et al. Comprehensive Molecular Profiling of Desmoplastic Small Round Cell Tumor. *Mol Cancer Res* (2021) 19(7):1146–55. doi: 10.1158/1541-7786.MCR-20-0722
47. Jamshidi F, Bashashati A, Shumansky K, Dickson B, Gokgoz N, Wunder JS, et al. The Genomic Landscape of Epithelioid Sarcoma Cell Lines and Tumours. *J Pathol* (2016) 238(1):63–73. doi: 10.1002/path.4636
48. Hirozane T, Masuda M, Sugano T, Sekita T, Goto N, Aoyama T, et al. Direct Conversion of Osteosarcoma to Adipocytes by Targeting TNIK. *JCI Insight* (2021) 6(3):e137245. doi: 10.1172/jci.insight.137245
49. Janeway KA, Grier HE. Sequelae of Osteosarcoma Medical Therapy: A Review of Rare Acute Toxicities and Late Effects. *Lancet Oncol* (2010) 11(7):670–8. doi: 10.1016/S1470-2045(10)70062-0
50. Pang B, Hao Y. Integrated Analysis of the Transcriptome Profile Reveals the Potential Roles Played by Long Noncoding RNAs in Immunotherapy for Sarcoma. *Front Oncol* (2021) 11:690486. doi: 10.3389/fonc.2021.690486
51. Geier B, Kurmashev D, Kurmasheva RT, Houghton PJ. Preclinical Childhood Sarcoma Models: Drug Efficacy Biomarker Identification and Validation. *Front Oncol* (2015) 5:193. doi: 10.3389/fonc.2015.00193
52. Pettini F, Visibelli A, Cicaloni V, Iovinelli D, Spiga O. Multi-Omics Model Applied to Cancer Genetics. *Int J Mol Sci* (2021) 22(11):5751. doi: 10.3390/ijms22115751
53. Chakraborty S, Hosen MI, Ahmed M, Shekhar HU. Onco-Multi-OMICS Approach: A New Frontier in Cancer Research. *BioMed Res Int* (2018) 2018:9836256. doi: 10.1155/2018/9836256
54. Shen R, Olshen AB, Ladanyi M. Integrative Clustering of Multiple Genomic Data Types Using a Joint Latent Variable Model With Application to Breast and Lung Cancer Subtype Analysis. *Bioinformatics* (2009) 25(22):2906–12. doi: 10.1093/bioinformatics/btp543
55. Koh HWL, Fermin D, Vogel C, Choi KP, Ewing RM, Choi H. Iomicspass: Network-Based Integration of Multiomics Data for Predictive Subnetwork Discovery. *NPJ Syst Biol Appl* (2019) 5:22. doi: 10.1038/s41540-019-0099-y
56. Miallot R, Galland F, Millet V, Blay JY, Naquet P. Metabolic Landscapes in Sarcomas. *J Hematol Oncol* (2021) 14(1):114. doi: 10.1186/s13045-021-01125-y
57. Esperança-Martins M, Fernandes I, Soares do Brito J, Macedo D, Vasques H, Serafim T, et al. Sarcoma Metabolomics: Current Horizons and Future Perspectives. *Cells* (2021) 10(6):1432. doi: 10.3390/cells10061432
58. Min L, Choy E, Tu C, Hornicek F, Duan Z. Application of Metabolomics in Sarcoma: From Biomarkers to Therapeutic Targets. *Crit Rev Oncol Hematol* (2017) 116:1–10. doi: 10.1016/j.critrevonc.2017.05.003
59. Thoenen E, Curl A, Iwakuma T. TP53 in Bone and Soft Tissue Sarcomas. *Pharmacol Ther* (2019) 202:149–64. doi: 10.1016/j.pharmthera.2019.06.010
60. Toguchida J, Yamaguchi T, Ritchie B, Beauchamp RL, Dayton SH, Herrera GE, et al. Mutation Spectrum of the P53 Gene in Bone and Soft Tissue Sarcomas. *Cancer Res* (1992) 52(22):6194–9.
61. Grünewald TG, Alonso M, Avnet S, Banito A, Burdach S, Cidre-Aranaz F, et al. Sarcoma Treatment in the Era of Molecular Medicine. *EMBO Mol Med* (2020) 12(11):e11131. doi: 10.15252/emmm.201911131

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fonc.2022.952228

OPEN ACCESS

EDITED BY
Jilong Yang,
Tianjin Medical University Cancer Institute and Hospital, China

REVIEWED BY
Lisha Qi,
Tianjin Medical University Cancer Institute and Hospital, China
Haixiao Wu,
Tianjin Medical University Cancer Institute and Hospital, China

*CORRESPONDENCE
Li Min
jacky-min@163.com
Chongqi Tu
tucq@scu.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 24 May 2022
ACCEPTED 27 June 2022
PUBLISHED 22 July 2022

CITATION
He X, Lu M, Hu X, Li L, Zou C, Luo Y, Zhou Y, Min L and Tu C (2022) Osteosarcoma immune prognostic index can indicate the nature of indeterminate pulmonary nodules and predict the metachronous metastasis in osteosarcoma patients. *Front. Oncol.* 12:952228. doi: 10.3389/fonc.2022.952228

Osteosarcoma immune prognostic index can indicate the nature of indeterminate pulmonary nodules and predict the metachronous metastasis in osteosarcoma patients

Xuanhong He†, Minxun Lu†, Xin Hu†, Longqing Li, Chang Zou, Yi Luo, Yong Zhou, Li Min* and Chongqi Tu*

Department of Orthopedics, Orthopaedic Research Institute, West China Hospital, Sichuan University, Chengdu, China

Purpose: The relationship between indeterminate pulmonary nodules (IPNs) and metastasis is difficult to determine. We expect to explore a predictive model that can assist in indicating the nature of IPNs, as well as predicting the probability of metachronous metastasis in osteosarcoma patients.

Patients and methods: We conducted a retrospective study including 184 osteosarcoma patients at West China Hospital from January 2016 to January 2021. Hematological markers and clinical features of osteosarcoma patients were collected and analyzed.

Results: In this study, we constructed an osteosarcoma immune prognostic index (OIPI) based on the lung immune prognostic index (LIPI). Compared to other hematological markers and clinical features, OIPI had a better ability to predict metastasis. OIPI divided 184 patients into four groups, with the no-OIPI group (34 patients), the light-OIPI group (35 patients), the moderate-OIPI group (75 patients), and the severe-OIPI group (40 patients) ($P < 0.0001$). Subgroup analysis showed that the OIPI could have a stable predictive effect in both the no-nodule group and the IPN group. Spearman's rank correlation test and Kruskal–Wallis test demonstrated that the OIPI was related to metastatic site and metastatic time, respectively. In addition, patients with IPNs in high-OIPI (moderate and severe) groups were more likely to develop metastasis than those in low-OIPI (none and light) groups. Furthermore, the combination of OIPI with IPNs can more accurately identify patients with metastasis, in which the high-OIPI group had a higher metastasis rate, and the severe-OIPI group tended to develop metastasis earlier than the no-OIPI group. Finally, we constructed an OIPI-based nomogram to predict 3- and 5-year metastasis rates. This nomogram could bring net benefits for more patients according to the decision curve analysis and clinical impact curve.

Conclusion: This study is the first to assist chest CT in diagnosing the nature of IPNs in osteosarcoma based on hematological markers. Our findings suggested that the OIPI was superior to other hematological markers and that OIPI can act as an auxiliary tool to determine the malignant transformation tendency of IPNs. The combination of OIPI with IPNs can further improve the metastatic predictive ability in osteosarcoma patients.

KEYWORDS

osteosarcoma, inflammation, osteosarcoma immune prognostic index, metastatic predictive markers, indeterminate pulmonary nodules

Introduction

Osteosarcoma is the most common primary bone malignancy primarily affecting children, adolescents, and the elderly (1). Current standard treatment for primary osteosarcoma includes neoadjuvant chemotherapy, wide surgical resection and adjuvant chemotherapy (1, 2). As comprehensive treatment advances, the 5-year overall survival (OS) rate improves to 60%–70%, while it decreases to 20%–30% when metastasis occurs (3). Metastasis remains the biggest obstacle to the clinical outcome of osteosarcoma (3, 4). Almost all osteosarcoma patients have subclinical micrometastatic disease at the time of initial diagnosis; however, metastatic status can be detected in only 20% of patients (5, 6). Patients with subclinical micrometastases frequently develop metastatic disease during follow-up, mainly leading to clinical treatment failure and a fatal clinical course (7–9).

The lung is the main metastatic site in patients with osteosarcoma (3, 10). An accurate evaluation of the lung metastatic status is essential. In the early stages of metastasis, lung metastasis always presents as micrometastasis; these micrometastases are difficult to distinguish from other benign nodules (3, 11, 12). Those benign and malignant undetermined pulmonary nodules are called indeterminate pulmonary nodules (IPNs); they are defined as non-calcified nodules with a maximum diameter <10 mm (13–15). With the application of fine-section computed tomography (CT), more suspicious pulmonary nodules are detected, bringing new challenges for accurate identification of pulmonary metastasis status in osteosarcoma patients (16). Since IPNs are not specific in cancer patients, the identification of their nature is difficult or even not feasible (11, 12, 16, 17). Due to the size of IPNs, needle biopsy is usually not feasible, and as an invasive test, biopsy may be excessive (17, 18). Clinically, radiologists often estimate the probability of lung metastasis with nodule size, margin, presence of calcification, and nodule amount (19–21). However, available studies suggest that none of these features could adequately distinguish malignancy from benign lesions (11). Therefore, more markers need to be included to assist in the judgment of the nature of IPNs.

Timely determination of the nature of IPNs and accurate development of individualized treatment decisions could improve the prognosis of osteosarcoma patients (3, 11, 17). In recent years, researchers have tried to determine the nature of IPNs by cell-free DNA (cfDNA), microRNA (miRNA), and circulating tumor cells (CTCs) (22). These specimens have the advantage of being non-invasive and reproducible (22). However, due to the limitation of sensitivity, specificity, and high cost, these biomarkers cannot be applied in clinical practice. In addition, an increasing number of studies have shown that hematological markers (such as the neutrophil–lymphocyte ratio (NLR), platelet–lymphocyte ratio (PLR), lymphocyte–monocyte ratio (LMR), and serum lactate dehydrogenase (LDH)) are associated with the prognosis of various cancers, including osteosarcoma (23–28). More surprisingly, some hematological markers, such as the lung immune prognostic index (LIPI), can predict the response to immunotherapy by reflecting the proinflammatory status (29–33). Therefore, it is reasonable to speculate that these hematological markers could assist in judging the nature of IPNs.

To the best of our knowledge, there have been no studies applying hematological markers to assist in the judgment of the nature of IPNs in osteosarcoma. Therefore, the main purpose of this study was to evaluate the value of classical hematological markers such as NLR, PLR, and LMR and the combination of hematological markers of LIPI in the judgment of the nature of IPNs. In addition, we modified the construction of the OIPI based on the LIPI and assessed its ability to predict the nature of IPNs.

Patients and methods

Patients

After obtaining institutional review board approval, we retrospectively reviewed the clinical data of osteosarcoma

patients from January 2016 to January 2021 in the database of the Musculoskeletal Tumor Center of West China Hospital. The inclusion criteria were as follows (1): patients with high-grade osteosarcoma confirmed by histopathology; 2) patients with complete hematological test results and staging chest CT prior to the neoadjuvant chemotherapy; and 3) patients who received standard treatment at West China Hospital. The exclusion criteria were as follows: 1) patients who had received neoadjuvant chemotherapy before their first-time consultancy in our hospital; 2) patients with hematological diseases and other malignancies (3); patients who did not receive staging chest CT during the follow-up; and (4) patients with distal metastasis at the time of diagnosis. Finally, a total of 184 patients were included in our study. Metastasis-free survival (MFS) was calculated from the date of diagnosis to the date of metastasis or last follow-up. Each patient was regularly followed up until death or January 2022. All the patients obeyed the follow-up rules: reexamination every 3 months within 1 year after surgery; every 4 months for 1–2 years after surgery; every 5 months for 2–3 years after surgery; twice a year for 3–5 years after surgery; and yearly after 5 years postoperatively.

Data collection and processing

Leukocyte count (Leut#), neutrophil count (Neut#), lymphocyte count (LYMPH#), monocyte count (MONO#), platelet count (PLT), lactate dehydrogenase (LDH), hydroxybutyrate dehydrogenase (HBDH), creatine kinase (CK), and alkaline phosphatase (ALP) were extracted from the blood routine of the 184 patients prior to neoadjuvant chemotherapy. The formulas for calculating NLR, PLR, LMR, and dNLR are as follows: NLR = Neut#/LYMPH#, PLR = PLT/LYMPH#, LMR = LYMPH#/MONO#, and dNLR= Neut#/(Leut#-Neut#). In addition, age, gender, tumor site, pathologic fracture status, and tumor metastasis status were collected from the patients' medical records. In the overall cohort, the optimal cutoff value for each hematological marker was calculated based on the time-dependent receiver operating curve (ROC) and converted into a binary variable according to the cutoff value.

Establishment and validation of the OIPI

Referring to the development of the LIPI, we established the prognostic model OIPI by combining the hematological indexes with a higher area under curve (AUC) in the ROC curves according to our results. Then, we compared the prognostic predictive effect of the OIPI with that of other hematological factors and clinical characteristics by time-dependent ROC. To verify whether the OIPI is an independent risk factor for metastasis in osteosarcoma patients, we conducted univariate and multivariate analyses. Significant factors in univariate analyses were then subjected to multivariate analyses to determine independent risk factors for metastasis. Furthermore, the association between the OIPI and metastatic sites or metachronous metastasis time was also explored by Spearman's rank correlation analysis, Kaplan–Meier survival analysis, and Kruskal–Wallis test.

Identification of IPNs

Computed tomography scans and reports were reviewed by at least two radiologists. Rissing et al. (13), defined IPNs as non-calcified nodules <10 mm in maximal diameter; according to this, all osteosarcoma patients were classified as no-nodule and IPNs at the time of diagnosis. All the patients received staging chest CT during the follow-up. Patients were considered to have pulmonary metastases when the following first occurred: maximum diameter of a pulmonary nodule increased by at least 25%, subsequent appearance of new pulmonary nodules on chest CT during follow-up, nodules were pathologically diagnosed as metastases, the date the treating oncologist documented the presence of pulmonary metastasis, or the date the oncologist documented the occurrence of pulmonary metastases.

Construction and validation of the OIPI-based nomogram

After a multistep screening process, an osteosarcoma metachronous metastasis nomogram was constructed combining the OIPI and clinical features. For each osteosarcoma patient, the total point was equal to the sum of the points of all metastasis prediction factors. The relationships between the total points and the probability of MFS are shown at the bottom of the nomogram. We evaluated the discrimination ability and accuracy of the nomogram by Harrell's concordance index and calibration curve, respectively. In the calibration curve, the diagonal acts as a reference line and represents the best prediction. Decision curve analysis (DCA) was used to evaluate the clinical application of the nomogram by estimating the net benefits at different threshold probabilities. The clinical impact curve was also drawn to predict reduction intervention probability per 100 patients.

Statistical analysis

The Kolmogorov–Smirnov test was used to assess whether continuous variables were normally distributed, and the Mann–Whitney U test or Spearman correlation analysis was used to assess differences between continuous variables according to the results. Categorical variables were evaluated using the chi-square test and Fisher's exact test based on the number of individuals in

each group. All statistical analyses were conducted using R software, version 4.1.0 (Institute for Statistics and Mathematics, Vienna, Austria). P-values < 0.05 were considered to indicate statistical significance.

Results

Patient characteristics and optimal cutoff values of hematological factors

The patients included in this study consisted of 107 men and 77 women. The age of the patients ranged from 7 to 67 years, with a mean age of 21 years. Tumors were mainly located at the extremities in 176 patients, and only eight patients had tumors affecting the extra-extremities. Pathological fracture at the time of diagnosis and metachronous metastasis were found in 19 and 64 patients respectively. The time to develop metachronous metastasis in the 64 patients ranged from 2 to 52 months with a mean time of 14.7 months. Among 184 osteosarcoma patients, the average MFS was 30.5 months. In addition, according to the initial chest CT, 67 of 184 patients were classified as having IPNs and 117 of 184 patients were classified as having no nodule. In 64 patients who developed metachronous metastasis, 32 patients had no nodule and 32 patients had IPNs (**Table 1**). The optimal cutoff values for hematological markers are also shown in **Supplementary Table 1** (NLR, PLR, LMR, LDH, dNLR, HBDH, CK, ALP).

We developed the LIPI with LDH and dNLR according to a previous study (30). Surprisingly, we found that HBDH was also a significant predictive factor in osteosarcoma with a high AUC value in t-ROC. Thus, we constructed the osteosarcoma immune prognostic index (OIPI) combining LIPI and HBDH (**Figure 1A**). As shown in **Figures 1A**, the t-ROC analysis demonstrated that the AUC of the OIPI was larger than that of inflammatory markers (dNLR, LDH, HBDH, LIPI) and clinical features (gender, age, tumor site, pathological fracture, and IPNs) (**Figures 1A, B**). This result showed that the OIPI performed better in predicting the metastasis than other hematological factors and clinical features. The OIPI divided 184 patients into four groups according to the cutoff values of hematological factors, with the no-OIPI group for 34 patients, light-OIPI group for 35 patients, moderate-OIPI group for 75 patients, and severe-OIPI group for 40 patients (P < 0.0001). For example, a patient with high dNLR, high LDH, and high HBDH was thought to have three high cutoff values and was categorized as severe OIPI (**Figure 2**).

Univariate analysis and multivariate analysis were conducted to further explore the correlation between predictive factors and metastasis in 184 patients. According to the univariate analysis, the pathological fracture (hazard ratio (HR) 2.006; [95% confidence interval CI] 1.018–3.953, P = 0.044), IPNs (HR 2.291 [95% CI] 1.398–3.756, P = 0.001), NLR (HR 2.182 [95% CI] 1.331–3.578, P = 0.002), PLR (HR 2.289 [95% CI] 1.363–3.845, P = 0.002), ALP (HR 2.189 [95% CI] 1.316–3.64, P = 0.003), and OIPI (HR 2.140 [95% CI] 1.590–2.88), P < 0.001) were associated with metastasis and were selected to perform multivariate analysis to identify independent risk factors for metastasis. The multivariate analysis revealed that IPNs (HR 1.717 [95% CI] 1.022–2.887, P = 0.041), PLR (HR 2.185 [95% CI] 1.247–3.829, P = 0.006), and OIPI (HR 1.950 [95% CI] 1.336–2.846, P < 0.001) were independent risk factors for metastasis (**Figures 2B**).

Effect of OIPI in evaluating the metastatic sites and metastatic time of patients with metachronous metastasis in 184 osteosarcoma patients

We further explored the clinical significance of the OIPI in patients developing metachronous metastasis. Spearman's correlation analysis was applied to explore the relationship between OIPI and metastasis sites (**Figure 2D**). The results demonstrated that the OIPI was significantly associated with the metastasis sites, in which more patients developed lung metastasis and extrapulmonary metastasis in the high-OIPI group (moderate and severe) than in the low-OIPI group (none and light). The Kruskal–Wallis test was used to compare differences in metachronous metastases in the four OIPI groups (**Figure 2E**). As shown, compared with the no-OIPI group, the moderate-OIPI group and the severe-OIPI group developed metachronous metastases earlier (P = 0.016 and P = 0.017, respectively).

Effect of the OIPI in predicting metachronous metastasis in patients with IPNs or without IPNs

To evaluate the stability of the OIPI and improve its accurate clinical application, we explored the application of the OIPI in the subgroup with IPNs (36.4%) or no nodule (63.6%). In patients with IPNs, the OIPI divided patients into four groups with the no-OIPI group for 9, light-OIPI group for 12, moderate-OIPI group for 28, and severe-OIPI group for 18 (**Figure 3A**) (P = 0.0044). According to the results of univariate analysis and multivariate analysis, the OIPI was the independent risk factor for metachronous metastasis (**Figures 3B,C**). These results indicated that the OIPI was a stable tool in predicting the metachronous metastasis. In addition, Spearman's rank correlation test demonstrated that OIPI was related to metastatic sites in patients with IPNs. Patients with IPNs in moderate or severe OIPI groups developed more lung metastases (**Figure 3D**).

In patients with no nodule, the OIPI divided patients into four groups with the no-OIPI group for 25, light-OIPI group for 23, moderate-OIPI group for 47, and severe-OIPI group for 22

TABLE 1 Characteristics of 184 osteosarcoma patients.

Characteristics	Patients, n (%)		P-value
	Non-metastasis(n = 120)	Metachronous metastasis(n = 64)	
Age (years)			
>22	35 (29.2%)	19 (29.7%)	1.000
≤22	85 (70.8%)	45 (70.3%)	
Gender			
Male	54 (45.0%)	41 (64.1%)	0.273
Female	66 (55.0%)	23 (35.9%)	
Tumor site			
Extremities	116 (96.7%)	60 (93.8%)	0.452
Non-extremities	4 (3.3%)	4 (6.2%)	
Pathological fracture			
Yes	9 (7.5%)	10 (15.6%)	0.125
No	111 (92.5%)	54 (84.4%)	
Chest CT			
No-nodule	85 (70.8%)	32 (50.0%)	0.006
IPNs	35 (29.2%)	32 (50.0%)	
NLR			
Low	83 (69.2%)	28 (43.8%)	0.001
High	37 (30.8%)	36 (56.2%)	
PLR			
Low	100 (83.3%)	42 (65.6%)	0.009
High	20 (16.7%)	22 (34.4%)	
LMR			
Low	78 (65.0%)	48 (75.0%)	0.001
High	42 (35.0%)	16 (25.0%)	
LDH			
Low	51 (42.5%)	13 (20.3%)	0.003
High	69 (57.5%)	51 (79.7%)	
dNLR			
Low	85 (70.8%)	31 (48.4%)	0.004
High	35 (29.2%)	33 (51.6%)	
HBDH			
Low	58 (48.3%)	9 (14.1%)	0.000
High	62 (51.7%)	55 (85.9%)	

(Continued)

10.3389/fonc.2022.952228

TABLE 1 Continued

Characteristics	Patients, n (%)		P-value
	Non-metastasis(n = 120)	Metachronous metastasis(n = 64)	
CK			
Low	104 (86.7%)	48 (75.0%)	0.065
High	16 (13.3%)	16 (25.0%)	
ALP			
Low	67 (55.8%)	26 (40.6%)	0.063
High	53 (44.2%)	38 (59.4%)	
LIPI			
Good	36 (30.0%)	6 (9.4%)	0.000
Intermediate	64 (53.3%)	32 (50.0%)	
Poor	20 (16.7%)	26 (40.6%)	
OIPI			
None	31 (25.8%)	3 (4.7%)	0.000
Light	27 (22.5%)	8 (12.5%)	
Moderate	47 (39.2%)	28 (43.8%)	
Poor	15 (12.5%)	25 (39.0%)	

IPNs, indeterminate pulmonary nodules; NLR, neutrophil–lymphocyte ratio; PLR, platelet–lymphocyte ratio; LMR, lymphocyte–monocyte ratio; LDH, lactate dehydrogenase; dNLR, derived neutrophil to lymphocyte ratio; HBDH, hydroxybutyrate dehydrogenase; CK, creatine kinase; ALP, alkaline phosphatase; LIPI, lung immune prognostic index; OIPI, osteosarcoma immune prognostic index.

10.3389/fonc.2022.952228

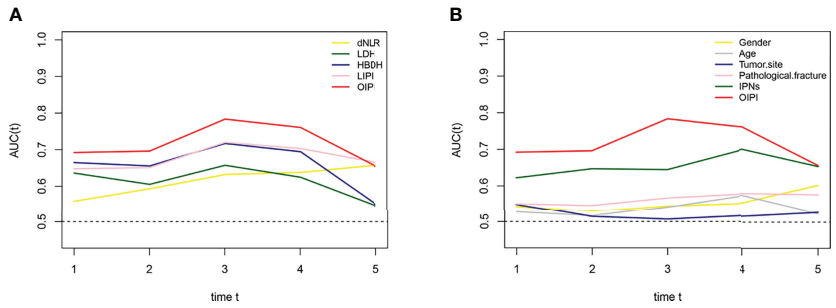


FIGURE 1
Comparison of different clinical biomarkers in predicting metachronous metastasis. **(A)** The difference in the predictive ability of different hematological markers is shown in the time-dependent ROC curve, in which a larger AUC value indicates a better metastatic predictive ability. **(B)** Difference in the predictive ability of different clinical features. dNLR, derived neutrophil-to-lymphocyte ratio; LDH, lactate dehydrogenase; HBDH, hydroxybutyrate dehydrogenase; LIPI, lung immune prognostic index; OIPI, osteosarcoma immune prognostic index; ROC, receiver operating curve; AUC, area under the curve; IPNs, indeterminate pulmonary nodules.

(Figure 3E) (P = 0.0013). According to the results of the univariate analysis and multivariate analysis, the PLR and OIPI were independent risk factors for metastasis in 117 no-nodule patients (Figures 3F,G). Similarly, the Spearman's rank correlation test demonstrated that OIPI was also related to metastatic sites in patients with no nodule. Patients with no

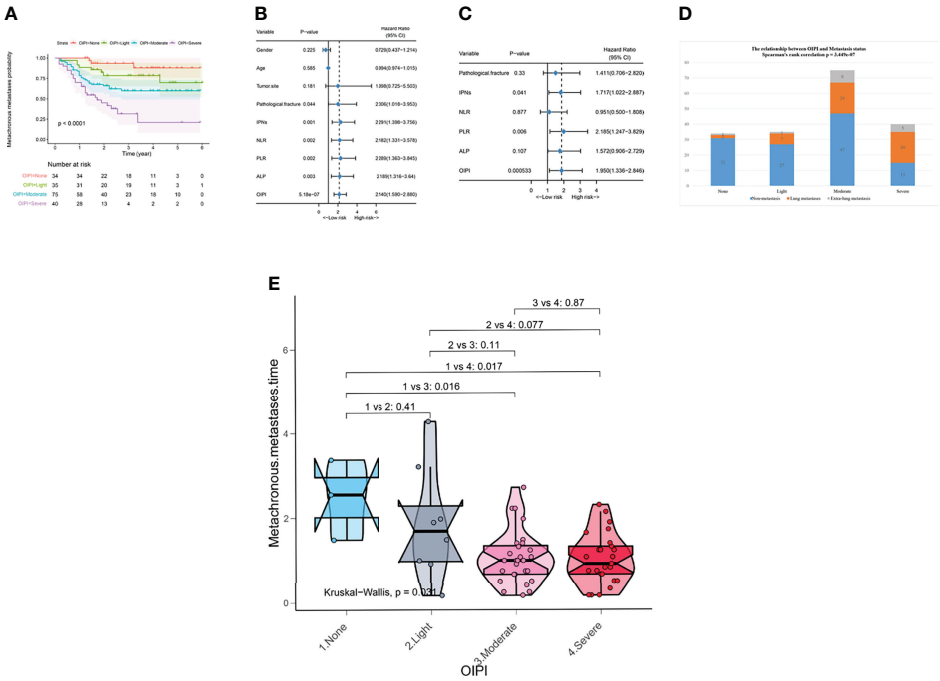


FIGURE 2
Effect of the OIPI in predicting metachronous metastasis, metastatic sites, and metastatic time in 184 patients. **(A)** OIPI divided 184 osteosarcoma patients into four groups. According to the logistic regression analysis, the differences between four OIPI groups in the metachronous metastasis probability were significant. **(B)** Forest plot showing the results of univariate Cox regression analysis of hematological markers and clinical features in 184 osteosarcoma patients. **(C)** Forest plot showing the results of multivariate Cox regression analysis of hematological markers and clinical features in 184 osteosarcoma patients. **(D)** Spearman's rank correlation test demonstrated that OIPI was related to metastatic sites. **(E)** Kruskal–Wallis test demonstrated that there was a difference in metastatic time among four groups (P = 0.031). Patients in moderate and severe OIPI groups developed metachronous metastasis earlier than that of the no-OIPI group (P = 0.016 and P = 0.017, respectively). OIPI, osteosarcoma immune prognostic index; IPNs, indeterminate pulmonary nodules; NLR, neutrophil–lymphocyte ratio; PLR, platelet–lymphocyte ratio; ALP, alkaline phosphatase; OIPI, osteosarcoma immune prognostic index.

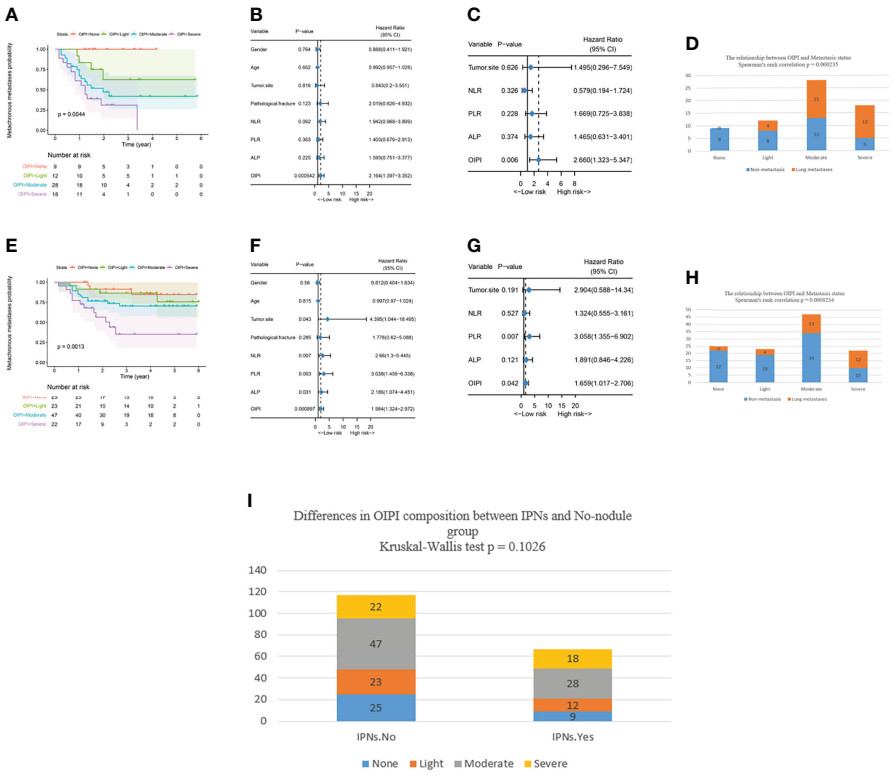


FIGURE 3
Effect of the OIPI in predicting metachronous metastasis in patients with no nodule or IPNs. **(A)** OIPI divided 67 patients with IPNs into four groups. There are significant differences among patients in the four groups (P = 0.0044). **(B)** Forest plot showing the results of univariate Cox regression analysis of hematological markers and clinical features in 67 patients with IPNs. **(C)** Forest plot showing the results of multivariate Cox regression analysis of hematological markers and clinical features in 67 patients with IPNs. **(D)** Spearman's rank correlation test demonstrated that the OIPI was related to metastasis site in patients with IPNs (P = 0.000235). **(E)** OIPI divided 117 patients with no nodule into four groups. There were significant differences among patients in the four groups (P = 0.0013) **(F)** Forest plot showing the results of univariate COX regression analysis of hematological markers and clinical features in 117 patients with no nodule. **(G)** Forest plot showing the results of multivariate Cox regression analysis of hematological markers and clinical features in 117 patients with no nodule. **(H)** Spearman's rank correlation test demonstrated that the OIPI was related to metastasis site in patients with no nodule (P = 0.0008234). **(I)** The Kruskal–Wallis test showed that there was no significant difference in the composition of OIPI between the IPN group and the no-nodule group. OIPI, osteosarcoma immune prognostic index; IPNs, indeterminate pulmonary nodules; NLR, neutrophil–lymphocyte ratio; PLR, platelet–lymphocyte ratio; ALP, alkaline phosphatase.

nodule in the moderate- or severe-OIPI groups developed more lung metastases (Figure 3H).

We also applied the Kruskal–Wallis test to analyze the difference in the composition of OIPI between the IPN group and the no-nodule group. The result shows that there is no significant difference in the composition of OIPI between the IPN group and the no-nodule group (Figure 3).

Effect of the combination of the OIPI and IPNs in predicting the MFS and metastatic time

We also explored the metastatic predictive function of the combination of the OIPI and IPNs in osteosarcoma patients. A total of 184 osteosarcoma patients were divided into eight groups according to the initial CT report (no-nodule or IPNs) and the OIPI classification. As shown in Figure 4A, in patients without nodules, group 4 had a higher probability of metastasis than group 1 (P = 0.003), group 2 (P = 0.0136), or group 3 (P = 0.0424). Among 184 osteosarcoma patients, patients in group 7 and group 8 were more likely to develop metastasis than patients in the other groups (group 1 vs. group 7, P = 0.003; group 1 vs. group 8, P < 0.0001; group 2 vs. group 7, P = 0.0136; group 2 vs. group 8, P < 0.0001; group 3 vs. group 7, P = 0.0302; group 3 vs. group 8, P = 0.001; group 5 vs. group 7, P = 0.031; group 5 vs. group 8, P = 0.0052; group 6 vs. group 8, P = 0.0424). Therefore, the patients in group 4, group 7, and group 8 were considered the metastasis high-risk groups. Then, we divided the patients into four groups according to the presence of IPNs (yes vs. not) and the OIPI (none and light (low OIPI) vs. moderate and severe (high OIPI)). As shown in Figures 4B,C, patients in group 3 and group 4 were the metastasis high-risk groups and always developed

10.3389/fonc.2022.952228

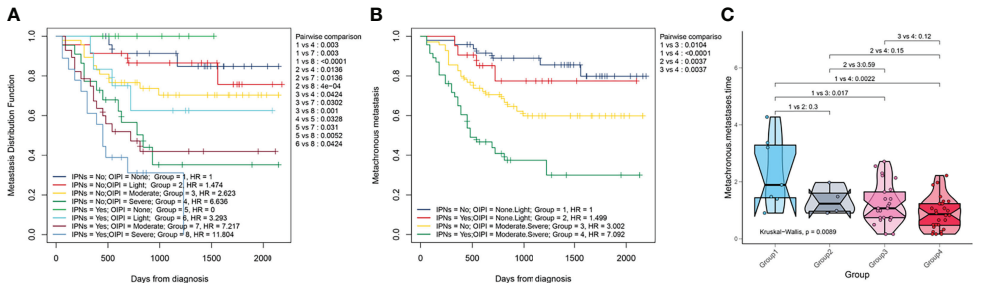


FIGURE 4
Effect of the combination of the OIPI and IPNs in predicting the MFS and metastatic time. **(A)** A total of 184 osteosarcoma patients were divided into eight groups according to the combination of OIPI and IPNs. The difference in metastatic predictive ability among eight groups was shown. **(B)** A total of 184 osteosarcoma patients were divided into four groups according to the combination of OIPI and IPNs. The difference in metastatic predictive ability among the four groups was shown. **(C)** The Kruskal–Wallis test demonstrated that there was a difference in metastatic time among the four groups (P = 0.0089). Patients in the moderate- and severe-OIPI groups developed metachronous metastasis earlier than those in the no-OIPI group (P = 0.017 and P = 0.0022, respectively). OIPI, osteosarcoma immune prognostic index; IPNs, indeterminate pulmonary nodules; MFS, metastasis-free survival.

metastasis earlier than patients in group 1 (P = 0.017 and P = 0.0022, respectively).

Construction and validation of the OIPI-based nomogram

To improve the clinical application of the OIPI, we constructed a nomogram combining the OIPI with clinical features. As shown in **Figure 5A**, Cox proportional hazards regression assigned a score based on the hazard ratio for each covariate, and the sum of the scores for each covariate was the nomogram total score. The C-index of this osteosarcoma metachronous metastasis nomogram was 0.72, and the calibration curve indicated that this nomogram could accurately predict 3- and 5-year OS (**Figure 5B**). Eventually, we also explored the clinical benefits of this nomogram with clinical DCA (**Figures 5C,D**). Our results demonstrate that the addition of this nomogram with the OIPI could bring significant net benefits over the model with only clinical features.

Discussion

In this study, we developed an osteosarcoma immune prognostic index (OIPI) for osteosarcoma patients with the combination of LDH, dNLR, and HBDH. The OIPI stratifies patients into four groups, none, light, moderate, and severe. The OIPI performs better in metastatic predictive ability than other hematological parameters and clinical features. Meanwhile, our results indicate that the OIPI is a stable predictive tool and could be used to evaluate the metastasis sites and MFS. Notably, this predictive model may help identify patients who develop early metachronous metastasis. Additionally, we also explored the predictive power of the combination of OIPI and IPNs; our results demonstrated that the OIPI could help clarify the nature of IPNs in chest CT. Also, the combination of OIPI and IPNs could help identify metastatic high-risk patients. Furthermore, we constructed a nomogram that had good predictive accuracy in predicting 3- and 5-year overall survival and is susceptible to bring significant net benefits to osteosarcoma patients.

Almost all patients with osteosarcoma have micrometastases at initial diagnosis, but only one-fifth of patients can be detected as metastasis status (3). In recent years, many efforts have been made to improve the prognosis of metastatic patients, such as the advancement of immune checkpoint inhibitors or TKI agents (34, 35). Unfortunately, several recent clinical trials have shown limited clinical benefit of these drugs for osteosarcoma (35, 36). Therefore, more focus on identifying micrometastases or early diagnosis of metastatic status may be an effective strategy. The lung is the most common site of osteosarcoma metastasis, and lung metastasis is associated with poor prognosis with a 5-year survival rate of 30%–40% (37–39).With the continuous development of CT detection technology, IPNs are detected in more osteosarcoma patients (16, 17, 40, 41), whereas there is still no consensus on the clinical relevance of IPNs and metastasis; it is currently based on nature (such as mineralization, round, and size greater than 5 mm) on CT alone to determine which IPNs will progress to lung metastases (13–15). Therefore, in clinical work, regular reexamination of CT is usually recommended for these patients. It is difficult to give personalized diagnosis and treatment opinions, meaning that patients with IPNs may miss the best time for treatment (11, 12, 16). In addition, although needle biopsy, thoracoscopy, and exploratory thoracotomy are considered to be options for defining the nature of nodules, the heterogeneity of IPNs renders their use limited due to inaccessibility to deep metastases, micrometastases, and

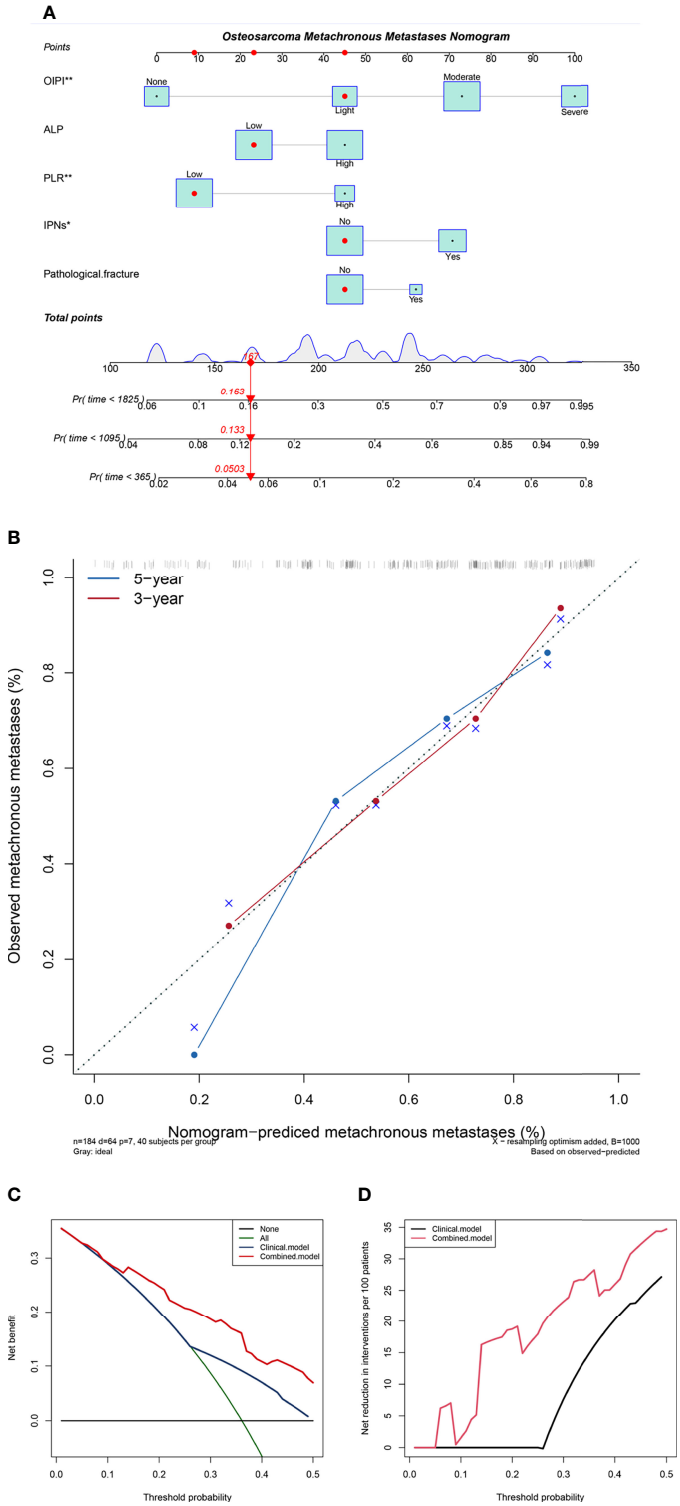


FIGURE 5
Construction and validation of the osteosarcoma metachronous metastasis nomogram. **(A)** The nomogram was constructed by combining OIPI, ALP, PLR, IPNs, and pathological fracture, and the sum of the scores for each covariate was the nomogram total score. **(B)** Calibration curves for the nomogram predicting 3- and 5-year metachronous metastasis of osteosarcoma patients. **(C)** The clinical net benefit curve of this nomogram. **(D)** Clinical net reduction curve for the nomogram. OIPI, osteosarcoma immune prognostic index; ALP, alkaline phosphatase; PLR, platelet–lymphocyte ratio; IPNs, indeterminate pulmonary nodules; *P < 0.05, **P < 0.01, ***P < 0.001.

invasiveness (11, 18). Therefore, this study expects to develop a predictive tool to assist in CT detection and make a personalized treatment plan for patients with IPNs, rather than recommending regular CT reviews to all patients.

An ideal prediction tool must be available, repeatable, predictable, and cost-effective. Recently, several novel assays such as lncRNAs have been developed. However, these methods are still some ways from clinical applications due to their high cost, difficulty in being obtained, and unified detection methods (42–45). Meanwhile, the predictive effect of hematological markers has been widely explored. A large number of hematological markers have been reported to have great predictive potential in osteosarcoma patients (23–28). Hematological markers could be ideal prediction tools because they are accessible to obtain and are reproducible. The LIPI is a novel hematological marker introduced by Mezquita et al., which is important for immunotherapeutic options and long-term survival prediction in advanced lung cancer and extrapulmonary cancer (30, 32, 33). The LIPI is composed of dNLR and LDH. Compared with the dNLR and LDH alone, the LIPI could better represent the inflammatory status and predict the prognosis of cancer patients (31). At present, the predictive role of the LIPI in osteosarcoma patients has not been reported. Given the important role of the LIPI in predicting prognosis and guiding immunotherapy selection, we hypothesized that the LIPI also has predictive potential of metastasis in osteosarcoma patients. Our results verified this hypothesis. In 184 osteosarcoma patients, the LIPI showed a better predictive ability of metastasis than dNLR or LDH alone (**Figure 1A**). At the same time, the results also showed that HBDH, an isoenzyme of LDH, had prognostic significance. The predictive ability of HBDH in predicting metastasis was even comparable to that of LIPI in the first 4 years after diagnosis (**Figure 1A**). Therefore, we combined the LIPI and HBDH to create the OIPI. The results showed that the predictive power of the OIPI was higher than that of other hematological markers and clinical natures (**Figures 1A,B**). Surprisingly, the predictive ability of the OIPI was even higher than that of the LIPI, implying that the OIPI may be more suitable for predicting metastasis in osteosarcoma patients. The possible reason is that the inclusion of HBDH promotes the OIPI to be a comprehensive inflammatory indicator, which helps the OIPI more fully reflect the inflammatory status and correlate with the metastatic status. On the other hand, the combination of dNLR, LDH, and HBDH can reduce potential bias because each individual index may be affected by various factors.

Excitingly, our results also revealed that the OIPI could predict metastatic sites and metastatic time. Patients in the moderate- and severe-OIPI groups were more likely to develop metachronous metastases, including lung and extra-lung metastases (**Figure 2E**). Therefore, for patients in the moderate- and severe-OIPI groups, both a more frequent chest CT and regular bone imaging or positron emission tomography-computed tomography (PET-CT) are recommended to timely detect extra-lung metastasis. (**Figure 2D**). In addition, patients in the severe-OIPI group may develop metastasis earlier than those in the no-OIPI group (**Figure 2E**). The reason may be that patients in the severe-OIPI group had a more severe inflammatory response, activation of pro-EMT signaling pathways, close interaction between immune cells and tumor cells, and more increased CTC concentrations. In that situation, tumor cells were more likely to colonize distant sites to form metastases (46, 47).

Considering the complexity of the tumor metastasis process, it is difficult to imagine that there is a test method that can independently and accurately predict tumor metastasis. Therefore, it is more important to combine OIPI with existing clinical features such as imaging features to more accurately predict metastasis in patients. Our results suggested that the OIPI can be used as a complementary tool for chest CT to synergistically evaluate the nature of IPNs. As shown in **Figure 3**, in both patients with IPNs and without nodules, the OIPI could evenly divide patients into four groups and was an independent predictor of metachronous metastasis, suggesting that the OIPI is a stable predictive tool. In addition, OIPI was associated with the metastatic site in patients with IPNs. In patients with IPNs, patients with a high OIPI had a higher probability to develop lung metastasis, revealing that the OIPI could assist in determining the nature of IPNs (**Figure 3D**). Furthermore, to investigate the predictive ability of metastasis in the OIPI combined with IPNs, we divided patients into different risk groups according to the OIPI and IPNs. In the high-risk group (moderate and severe OIPI groups), patients with IPNs had a higher probability of developing metastasis (**Figures 4A,B**) (group 7 vs. group 1, HR = 7.217, P = 0.003; group 8 vs. group 1, HR = 11.804, P < 0.0001). In addition, the combination of OIPI and IPNs also had some advantages in assessing the metastatic time. As shown, group 4 had an earlier onset of metastases than group 1 (P < 0.0001). As a result, we recommend that more attention should be paid to patients in the moderate- and severe-OIPI groups with IPNs. In these high-risk patients, long-term close follow-up, more frequent chest CT, or invasive surgery to identify metastases may be helpful for the early diagnosis of metastatic lesions. On the other hand, for patients in the no-OIPI group, the probability of malignant transformation of IPNs was low; thus, long-term follow-up and regular chest CT are necessary to avoid unnecessary invasive procedures (**Figures 4A,B**). Simply put, the OIPI can be used as a complementary tool of IPNs for predicting metastasis in clinical practice. For patients with IPNs, clinicians could combine the OIPI and chest CT to individualize the risk classification. Moreover, clinicians could develop individual treatment plans and offer accurate patient consultations.

Furthermore, we also constructed an OIPI-based nomogram for predicting the metachronous metastasis in osteosarcoma. A nomogram is an accurate and convenient mathematical model that can predict specific endpoints. It can help clinicians better diagnose and determine treatment options. Applying nomograms to assess

metastatic status is necessary (48). Nomograms are rarely reported in predicting metastasis in osteosarcoma. Most of the nomograms are established by integrating clinical parameters and genes associated with metastasis (48–51). However, due to the imprecision of clinical features and the clinical inapplicability of genes, the clinical application of these nomograms was limited. In the current study, we included the indicators significantly associated with metachronous metastasis including OIPI, initial CT report, and PLR in the construction of the nomogram (**Figure 5A**). At the same time, we also included ALP, pathological fracture, and other indicators considering their important prognostic role in osteosarcoma. According to the patients' individual information and corresponding value, we can obtain a total score to predict the risk of metachronous metastasis. Our results revealed that this OIPI-based nomogram can accurately predict the 3- and 5-year metastasis rates of osteosarcoma patients (**Figure 5B**). Additionally, compared with the prediction model without OIPI, the OIPI-based nomogram can benefit more osteosarcoma patients in the diagnosis and treatment process (**Figures 5C,D**). Based on the lack of metastasis prediction tools in clinical practice, we believe that this nomogram could be a good prediction tool for metachronous metastasis in osteosarcoma patients.

The mechanism of the OIPI in predicting metastasis may because it can reflect the extent of the inflammatory response and evaluate the metastasis status. As is well known, metastasis originates from the invasion of cancer cells from the epithelial layer to the surrounding tissue and the acquisition of the EMT (epithelial to mesenchymal transition) phenotype. Then the cancer cells could break through the basement membrane, invade the tissue, and reach lymphatic vessels or blood vessels for further spread (46, 47). Inflammation affects cancer invasion, EMT, and cell migration at various levels (52). Cytokines, tumor-specific inflammatory signals, and immune cells promote the migration of tumor cells and the establishment of a metastatic microenvironment by interacting with cancer cells or the microenvironment (52). Inflammatory cells such as monocytes and neutrophils can further aid in adhesion and extravasation processes, which will accelerate the adhesion of metastatic seeds, form complexes with cancer cells, regulate the adhesion and translocation throughout the vessel wall, and finally establish and maintain a metastatic niche (53–55). Oligocellular complexes between cancer cells themselves or immune cells–cancer cells also help to protect these metastatic seeds from immune surveillance (56). Platelets protect CTCs from lethal attack by the immune system or other pro-apoptotic stimuli and provide signals to establish a pro-metastatic niche environment that ultimately promotes tumor growth and metastasis (57). LDH, as a classical prognostic predictor of osteosarcoma, often detects higher LDH levels in metastatic patients than in non-metastatic patients (58, 59).At the same time, some clinical studies have also shown that an increase in lactate concentration is also associated with the subsequent development of nodules or distant metastasis (60). This shows the value of detecting LDH levels in predicting metastasis. As the cornerstone of OIPI, dNLR, LDH, and HBDH are all associated with metastasis. The constructed OIPI may reflect the extent of inflammation affecting cancer cell invasion, EMT, and cell migration at different levels, thereby assessing the possibility of patient metastasis.

It must be acknowledged that our study has several limitations. First, this single-center and retrospective study may have resulted in selection bias. Second, the results of the relationship between OIPI and metastatic and metastasis sites need to be interpreted with caution. Since there were only three cases of metastasis in the no-OIPI group, this may lead to instability in exploring the relationship between the OIPI and metastatic time. In the next stage, we plan to conduct further studies with larger numbers of participants and longer follow-up to further verify the clinical significance of the OIPI in predicting the metastatic time. Finally, the prognostic value of HBDH in osteosarcoma still needs further validation. In this study, we preliminarily investigated the prognostic value of HBDH, the isozyme of LDH. Surprisingly, HBDH behaved comparably to LDH in our cohort. However, studies on the prognostic value of HBDH in cancer patients are very scarce. In osteosarcoma, only our study reported the prognostic value of HBDH. Therefore, further studies are needed to elucidate the predictive ability of HBDH in patients with osteosarcoma or even cancer patients. Despite the limitations, our study is the first to interrogate the clinical significance of hematological markers with IPNs, providing a new idea for subsequent studies. Further validation of the OIPI in relation to metastatic time and treatment effect is needed. It is even more important to explore whether the OIPI can truly produce a net clinical benefit. For example, for patients with a high OIPI, whether closer follow-up and more frequent chest CT can benefit patients or not needs to be further determined.

Conclusion

In conclusion, this study is the first to assist chest CT in diagnosing the nature of IPNs in osteosarcoma based on hematological markers. Our findings suggest that an OIPI with a LIPI is superior to other hematological markers, and the OIPI can be used as an auxiliary tool to determine the malignant transformation tendency of IPNs. The combination of the OIPI with IPNs can further improve the metastatic predictive ability in osteosarcoma patients. Further studies are needed to validate our findings.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital, Sichuan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

XH, ML, and CT designed the research study. ML and LL performed the research. CZ provided help and advice on revising the manuscript. YL analyzed the data. XH and LM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Funding

This study is funded by the Science and Technology Research Program of Sichuan Province (2020YFS0036), and 1·3·5 project for disciplines of excellence, West China Hospital, Sichuan University (ZYJC18036).

Acknowledgments

Thanks to the support of West China Hospital, Sichuan University, for this research.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.952228/full#supplementary-material

References

1. Belayneh R, Fourman MS, Bhogal S, Weiss KR. Update on osteosarcoma. *Curr Oncol Rep* (2021) 23(6):71. doi: 10.1007/s11912-021-01053-7

2. Ritter J, Bielack SS. Osteosarcoma. *Ann Oncol* (2010) 21 Suppl 7:vii320–5. doi: 10.1093/annonc/mdq276

3. Sheng G, Gao Y, Yang Y, Wu H. Osteosarcoma and metastasis. *Front Oncol* (2021) 11:780264. doi: 10.3389/fonc.2021.780264

4. Wang W, Ding H, Sun Z, Jin C, Zhu Y, Wang X. A population-based propensity-matched study of regional dissections in patients with metastatic osteosarcoma. *J orthopaedic Surg Res* (2020) 15(1):107. doi: 10.1186/s13018-020-01592-y

5. Kaste SC, Pratt CB, Cain AM, Jones-Wallace DJ, Rao BN. Metastases detected at the time of diagnosis of primary pediatric extremity osteosarcoma at diagnosis: Imaging features. *Cancer* (1999) 86(8):1602–8. doi: 10.1002/(sici)1097-0142(19991015)86:8<1602::aid-cncr31>3.0.co;2-r

6. Harting MT, Blakely ML. Management of osteosarcoma pulmonary metastases. *Semin Pediatr surg* (2006) 15(1):25–9. doi: 10.1053/j.sempedsurg.2005.11.005

7. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. *Nat Rev Canc* (2014) 14(11):722–35. doi: 10.1038/nrc3838

8. Li SW, Hu KZ, Chen SC, Liu SL, Wang YH. High expression of long non-coding RNA LOC730101 correlates with distant metastasis and exhibits a poor prognosis in patients with osteosarcoma. *Eur Rev Med Pharmacol Sci* (2018) 22(13):4115–20. doi: 10.26355/eurrev_201807_15403

9. Glasser DB, Lane JM, Huvos AG, Marcove RC, Rosen G. Survival, prognosis, and therapeutic response in osteogenic sarcoma. the memorial hospital experience. *Cancer*. (1992) 69(3):698–708. doi: 10.1002/1097-0142(19920201)69:3<698::aid-cncr2820690317>3.0.co;2-g

10. Meyers PA, Heller G, Healey JH, Huvos A, Applewhite A, Sun M, et al. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. *J Clin Oncol* (1993) 11(3):449–53. doi: 10.1200/jco.1993.11.3.449

11. Tsoi KM, Lowe M, Tsuda Y, Lex JR, Fujiwara T, Almeer G, et al. How are indeterminate pulmonary nodules at diagnosis associated with survival in patients with high-grade osteosarcoma? *Clin orthopaedics related Res* (2021) 479(2):298–308. doi: 10.1097/corr.0000000000001491.

12. Seher K, Saeed H, Kareem T, Baqari AS, Niazi I, Wali RM. Indeterminate pulmonary nodules in osteosarcoma are associated with increased risk of pulmonary metastasis. *J Pediatr hematol/oncol* (2021) 43(6):e826–31. doi: 10.1097/mph.0000000000002124

13. Rissing S, Rougraff BT, Davis K. Indeterminate pulmonary nodules in patients with sarcoma affect survival. *Clin orthopaedics related Res* (2007) 459:118–21. doi: 10.1097/BLO.0b013e31805d8606

14. Nakamura T, Matsumine A, Matsusaka M, Mizumoto K, Mori M, Yoshizaki T, et al. Analysis of pulmonary nodules in patients with high-grade soft tissue sarcomas. *PLoS One* (2017) 12(2):e0172148. doi: 10.1371/journal.pone.0172148

15. Nakamura T, Matsumine A, Niimi R, Matsubara T, Kusuzaki K, Maeda M, et al. Management of small pulmonary nodules in patients with sarcoma. *Clin Exp metastasis* (2009) 26(7):713–8. doi: 10.1007/s10585-009-9270-y

16. Ghosh KM, Lee LH, Beckingsale TB, Gerrand CH, Rankin KS. Indeterminate nodules in osteosarcoma: What's the follow-up? *Br J Cancer* (2018) 118(5):634–8. doi: 10.1038/bjc.2017.453.

17. Zhou C, Wang Y, Qian G, Li H, Yu W, Shen Z, et al. Clinical significance of indeterminate pulmonary nodules on the survival of 364 patients with nonmetastatic, high-grade, localized osteosarcoma: A 12-year retrospective cohort study. *J Surg Oncol* (2021) 123(2):587–95. doi: 10.1002/jso.26316

18. Callister ME, Baldwin DR. How should pulmonary nodules be optimally investigated and managed? *Lung Cancer (Amsterdam Netherlands)* (2016) 91:48–55. doi: 10.1016/j.lungcan.2015.10.018.

19. Brader P, Abramson SJ, Price AP, Ishill NM, Emily ZC, Moskowitz CS, et al. Do characteristics of pulmonary nodules on computed tomography in children with known osteosarcoma help distinguish whether the nodules are malignant or benign? *J Pediatr Surg* (2011) 46(4):729–35. doi: 10.1016/j.jpedsurg.2010.11.027.

20. Ciccarese F, Bazzocchi A, Ciminari R, Righi A, Rocca M, Rimondi E, et al. The many faces of pulmonary metastases of osteosarcoma: Retrospective study on 283 lesions submitted to surgery. *Eur J radiol* (2015) 84(12):2679–85. doi: 10.1016/j.ejrad.2015.09.022

21. Cipriano C, Brockman L, Romancik J, Hartemayer R, Ording J, Ginder C, et al. The clinical significance of initial pulmonary micronodules in young sarcoma patients. *J Pediatr hematol/oncol* (2015) 37(7):548–53. doi: 10.1097/mph.0000000000000393

22. Tao R, Cao W, Zhu F, Nie J, Wang H, Wang L, et al. Liquid biopsies to distinguish malignant from benign pulmonary nodules. *Thorac canc* (2021) 12 (11):1647–55. doi: 10.1111/1759-7714.13982

23. Li YJ, Yao K, Lu MX, Zhang WB, Xiao C, Tu CQ. Prognostic value of the c-reactive protein to albumin ratio: A novel inflammation-based prognostic indicator in osteosarcoma. *OncoTar Ther* (2017) 10:5255–61. doi: 10.2147/ott.s140560

24. Liu B, Huang Y, Sun Y, Zhang J, Yao Y, Shen Z, et al. Prognostic value of inflammation-based scores in patients with osteosarcoma. *Sci Rep* (2016) 6:39862. doi: 10.1038/srep39862

25. Koh CH, Bhoo-Pathy N, Ng KL, Jabir RS, Tan GH, See MH, et al. Utility of pre-treatment neutrophil-lymphocyte ratio and platelet-lymphocyte ratio as prognostic factors in breast cancer. *Br J canc* (2015) 113(1):150–8. doi: 10.1038/bjc.2015.183

26. Gu X, Sun S, Gao XS, Xiong W, Qin S, Qi X, et al. Prognostic value of platelet to lymphocyte ratio in non-small cell lung cancer: Evidence from 3,430 patients. *Sci Rep* (2016) 6:23893. doi: 10.1038/srep23893

27. Li Y, Wang C, Xu M, Kong C, Qu A, Zhang M, et al. Preoperative NLR for predicting survival rate after radical resection combined with adjuvant immunotherapy with CIK and postoperative chemotherapy in gastric cancer. *J Cancer Res Clin Oncol* (2017) 143(5):861–71. doi: 10.1007/s00432-016-2330-1

28. Pan QX, Su ZJ, Zhang JH, Wang CR, Ke SY. A comparison of the prognostic value of preoperative inflammation-based scores and TNM stage in patients with gastric cancer. *OncoTar Ther* (2015) 8:1375–85. doi: 10.2147/ott.s82437

29. Kazandjian D, Gong Y, Keegan P, Pazdur R, Blumenthal GM. Prognostic value of the lung immune prognostic index for patients treated for metastatic non-small cell lung cancer. *JAMA Oncol* (2019) 5(10):1481–5. doi: 10.1001/jamaoncol.2019.1747

30. Mezquita L, Auclin E, Ferrara R, Charrier M, Remon J, Planchard D, et al. Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. *JAMA Oncol* (2018) 4(3):351–7. doi: 10.1001/jamaoncol.2017.4771

31. Auclin E, Vuagnat P, Smolenschi C, Taieb J, Adeva J, Nebot-Bral L, et al. Association of the lung immune prognostic index with immunotherapy outcomes in mismatch repair deficient tumors. *Cancers* (2021) 13(15):3776. doi: 10.3390/cancers13153776.

32. Feng JF, Zhao JM, Chen S, Chen QX. Prognostic significance of the lung immune prognostic index in patients with resected esophageal squamous cell carcinoma. *Cancer Manage Res* (2021) 13:2811–9. doi: 10.2147/cmar.s298412

33. Obayashi K, Miki J, Fukuokaya W, Yanagisawa T, Kimura S, Tsuzuki S, et al. The prognostic value of the preoperative lung immune prognostic index in patients with urothelial bladder cancer undergoing radical cystectomy. *Int J Clin Oncol* (2022) 27(2):396–402. doi: 10.1007/s10147-021-02059-8

34. Jafari F, Javdansirat S, Sanaie S, Naseri A, Shamekh A, Rostamzadeh D, et al. Osteosarcoma: A comprehensive review of management and treatment strategies. *Ann Diagn pathol* (2020) 49:151654. doi: 10.1016/j.anndiagpath.2020.151654

35. Xie L, Xu J, Sun X, Tang X, Yan T, Yang R, et al. Apatinib for advanced osteosarcoma after failure of standard multimodal therapy: An open label phase II clinical trial. *oncol* (2019) 24(7):e542–50. doi: 10.1634/theoncologist.2018-0542

36. Grignani G, Palmerini E, Ferraresi V, D'Ambrosio L, Bertulli R, Asaftei SD, et al. Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: A non-randomised phase 2 clinical trial. *Lancet Oncol* (2015) 16(1):98–107. doi: 10.1016/s1470-2045(14)71136-2

37. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: An analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol* (2002) 20(3):776–90. doi: 10.1200/jco.2002.20.3.776

38. Janeway KA, Barkauskas DA, Krailo MD, Meyers PA, Schwartz CL, Ebb DH, et al. Outcome for adolescent and young adult patients with osteosarcoma: A report from the children's oncology group. *Cancer.* (2012) 118(18):4597–605. doi: 10.1002/cncr.27414

39. Picci P, Mercuri M, Ferrari S, Alberghini M, Briccoli A, Ferrari C, et al. Survival in high-grade osteosarcoma: Improvement over 21 years at a single institution. *Ann Oncol* (2010) 21(6):1366–73. doi: 10.1093/annonc/mdp502

40. Pass HI, Dwyer A, Makuch R, Roth JA. Detection of pulmonary metastases in patients with osteogenic and soft-tissue sarcomas: The superiority of CT scans compared with conventional linear tomograms using dynamic analysis. *J Clin Oncol* (1985) 3(9):1261–5. doi: 10.1200/jco.1985.3.9.1261

41. Parsons AM, Ennis EK, Yankaskas BC, Parker LAJr., Hyslop WB, Detterbeck FC. Helical computed tomography inaccuracy in the detection of pulmonary metastases: Can it be improved? *Ann Thorac Surg* (2007) 84 (6):1830–6. doi: 10.1016/j.athoracsur.2007.06.069.

42. Wang T, Ji F, Dai Z, Xie Y, Yuan D. Increased expression of microRNA-191 as a potential serum biomarker for diagnosis and prognosis in human osteosarcoma. *Cancer biomarkers: section A Dis markers* (2015) 15(5):543–50. doi: 10.3233/cbm-150493

43. Liu Y, Teng Z, Wang Y, Gao P, Chen J. Prognostic significance of survivin expression in osteosarcoma patients: A meta-analysis. *Med Sci Monit* (2015) 21::2877–85. doi: 10.12659/msm.894448

44. Li F, Cao L, Hang D, Wang F, Wang Q. Long non-coding RNA HOTTIP is up-regulated and associated with poor prognosis in patients with osteosarcoma. *Int J Clin Exp pathol* (2015) 8(9):11414–20.

45. Li LQ, Zhang LH, Yuan YB, Lu XC, Zhang Y, Liu YK, et al. Signature based on metabolic-related gene pairs can predict overall survival of osteosarcoma patients. *Cancer Med* (2021) 10(13):4493–509. doi: 10.1002/cam4.3984

46. Varga J, Greten FR. Cell plasticity in epithelial homeostasis and tumorigenesis. *Nat Cell Biol* (2017) 19(10):1133–41. doi: 10.1038/ncb3611

47. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell.* (2004) 118(3):285–96. doi: 10.1016/j.cell.2004.07.013

48. Lu S, Wang Y, Liu G, Wang L, Wu P, Li Y, et al. Construction and validation of nomogram to predict distant metastasis in osteosarcoma: A retrospective study. *J orthopaedic Surg Res* (2021) 16(1):231. doi: 10.1186/s13018-021-02376-8

49. Fu Y, Bao Q, Liu Z, He G, Wen J, Liu Q, et al. Development and validation of a hypoxia-associated prognostic signature related to osteosarcoma metastasis and immune infiltration. *Front Cell Dev Biol* (2021) 9:633607. doi: 10.3389/fcell.2021.633607

50. Chen B, Zeng Y, Liu B, Lu G, Xiang Z, Chen J, et al. Risk factors, prognostic factors, and nomograms for distant metastasis in patients with newly diagnosed osteosarcoma: A population-based study. *Front endocrinol* (2021) 12:672024. doi: 10.3389/fendo.2021.672024

51. Zheng D, Xia K, Yu L, Gong C, Shi Y, Li W, et al. A novel six metastasis-related prognostic gene signature for patients with osteosarcoma. *Front Cell Dev Biol* (2021) 9:699212. doi: 10.3389/fcell.2021.699212

52. Greten FR, Grivennikov SI. Inflammation and cancer: Triggers, mechanisms, and consequences. *Immunity.* (2019) 51(1):27–41. doi: 10.1016/j.immuni.2019.06.025

53. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell.* (2014) 158(5):1110–22. doi: 10.1016/j.cell.2014.07.013

54. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature.* (2019) 566(7745):553–7. doi: 10.1038/s41586-019-0915-y

55. Wolf MJ, Hoos A, Bauer J, Boettcher S, Knust M, Weber A, et al. Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation *via* the JAK2-Stat5 and P38mapk pathway. *Cancer Cell* (2012) 22(1):91–105. doi: 10.1016/j.ccr.2012.05.023

56. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-Producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* (2015) 522(7556):345–8. doi: 10.1038/nature14282

57. Haemmerle M, Stone RL, Menter DG, Afshar-Kharghan V, Sood AK. The platelet lifeline to cancer: Challenges and opportunities. *Cancer Cell* (2018) 33 (6):965–83. doi: 10.1016/j.ccell.2018.03.002

58. Bacci G, Longhi A, Ferrari S, Briccoli A, Donati D, De Paolis M, et al. Prognostic significance of serum lactate dehydrogenase in osteosarcoma of the extremity: Experience at rizzoli on 1421 patients treated over the last 30 years. *Tumori* (2004) 90(5):478–84. doi: 10.1177/030089160409000507

59. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK, et al. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res* (2000) 60(4):916–21.

60. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW, et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-Neck cancer. *Int J Radiat oncol biol phys* (2001) 51 (2):349–53. doi: 10.1016/s0360-3016(01)01630-3

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 05 September 2022
DOI 10.3389/fonc.2022.992738

OPEN ACCESS

EDITED BY
Shunbin Xiong,
University of Texas MD Anderson Cancer Center, United States

REVIEWED BY
Jovanka Gencel-Augusto,
University of Texas MD Anderson Cancer Center, United States
Bilian Jin,
Dalian Medical University, China

*CORRESPONDENCE
Shaoqing Ni
chgcp@zju.edu.cn
Jinhu Wang
wjh@zju.edu.cn

SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 13 July 2022
ACCEPTED 16 August 2022
PUBLISHED 05 September 2022

CITATION
Wu J, Shou X, Cai J, Mao J, Qian J, Wang J and Ni S (2022) Prognostic factors of pediatric pelvic and genitourinary rhabdomyosarcoma: An analysis based on SEER database. *Front. Oncol.* 12:992738. doi: 10.3389/fonc.2022.992738

Prognostic factors of pediatric pelvic and genitourinary rhabdomyosarcoma: An analysis based on SEER database

Jiheng Wu[1], Xinyi Shou[2], Jiabin Cai[3], Junqing Mao[3], Jianqin Qian[1], Jinhu Wang[3]* and Shaoqing Ni[1,4]*

[1]National Clinical Trial Institute, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou, China, [2]The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou, China, [3]Department of Surgical Oncology, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou, China, [4]Research Center for Clinical Pharmacy, Zhejiang University, Hangzhou, China

Background: Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcomas in children. This study aimed to investigate the prognostic factors of pelvic and genitourinary RMS in children and evaluate the survival outcomes of these children treated with or without radiation therapy (RT).

Methods: The Surveillance, Epidemiology, and End Results Program (SEER) database was required for children with pelvic and genitourinary RMS. Overall survival (OS) and cancer-specific survival (CSS) were analyzed using the Kaplan-Meier method, log-rank test, Cox proportional hazards models, and propensity score-matched analyses.

Results: For the 262 patients analyzed, the most common biological subtypes were embryonic (n=209, 79.8%) and alveolar (n=29, 11.1%). Patients with alveolar RMS had the worst prognosis ($P < 0.05$). The testis (n=122, 46.6%) was the most common location, followed by the urinary bladder (n=57, 21.8%) and prostate (n=48, 18.3%). Uterus RMS had the highest survival rate, followed by testis, urinary bladder, and prostate RMS. Favorable prognostic factors were age at diagnosis < 15 years, non-alveolar histological subtype, early tumor stage (localized/regional), specific sites (uterus and testis), and treatment (cancer-directed surgery and chemotherapy) ($P < 0.05$). Propensity score-matched analyses comparing the cohorts of patients treated with or without RT demonstrated no significant differences in prognostic survival (OS: P=0.872, CSS: P=0.713).

Conclusion: The nomogram constructed based on independent prognostic factors may accurately predict survival rates at 1 and 5 years. Surgery and adjuvant chemotherapy can be effective treatments, but RT fails to guarantee a survival benefit. Therefore, prospective trials evaluating RT for pediatric pelvic and genitourinary RMS are warranted.

KEYWORDS

pediatric rhabdomyosarcoma, prognostic survival analysis, retrospective study, radiation therapy, outcomes assessment

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents aged 0-19 years (1). Pelvic and genitourinary RMS accounts for approximately 27% of all pediatric RMS (2). The pelvis and genitourinary organs are close to the digestive, reproductive, and urinary organs. These adjacent organs might be affected during RMS treatments (surgery, iliac artery chemotherapy, and radiation therapy (RT)), which may result in unsatisfactory treatment outcomes (3–6). Although existing guidelines recommend RT for patients with RMS, there are no available studies mentioning the prognosis of children with pelvic and genitourinary RMS receiving RT. This study selected pediatric patients with pelvic and genitourinary RMS from the Surveillance, Epidemiology, and End Results Program database (SEER, 1975-2016). Clinical features (sex, age, race, tumor site, and pathological type) and treatment methods (surgery, iliac artery chemotherapy, and RT) were used to determine the prognostic factors and assess prognostic survival.

Methods

The SEER database of the National Cancer Institute covers 26% of the incidence and survival data from 17 population-based cancer registries in the United States (7). We identified and included all patients aged 0-19 from the SEER database 1975-2016 who were histologically diagnosed with RMS (International Classification of Disease for Oncology [ICD-O-3] code '8900/3: Rhabdomyosarcoma, NOS', '8901/3: Pleomorphic rhabdomyosarcoma,' '8902/3: mixed type rhabdomyosarcoma,' '8910/3: Embryonal rhabdomyosarcoma,' '8912/3: Spindle cell rhabdomyosarcoma,' '8920/3: Alveolar rhabdomyosarcoma,' '8921/3: rhabdomyosarcoma with ganglionic differentiation'). Primary site-specific codes included RMS originating in the retroperitoneal pelvic area. Patient data extracted from the SEER database included demographic, pathological, and clinical variables. The demographic variables included sex and age at diagnosis. Pathologic variables included tumor histologic subtype, primary site, and extent of disease, as evaluated using collaborative stage coding methods. Clinical variables included chemotherapy (yes/no), RT (yes/no), surgery (yes/no), overall survival (OS), and cancer-specific survival (CSS).

This study was exempt from local research ethics committee approval, considering that SEER data were de-identified and publicly available for research use.

Statistical analysis

OS was defined as the time from diagnosis to death due to any cause. CSS was defined as the time from diagnosis to death due to pelvic and genitourinary RMS. Median survival time was defined as the length of time when half of the patients died. Kaplan-Meier univariate analysis was performed to calculate the OS and CSS curves (8). Least absolute shrinkage and selection operator (LASSO) regression was performed to select the initial factors and prevent overfitting of the multifactorial models. Covariates were assessed using multivariable Cox proportional hazard regression models with corresponding 95% confidence intervals (CIs).

The performance and discriminative power of prognostic factors were assessed using the concordance index (C-index) values and receiver operating characteristic curve (AUC), which was then visualized as nomograms using the R package "rms" (9). Propensity score analysis was performed to minimize selection bias because of the retrospective nature of the data analysis (10). The 110 propensity score-matched cases were evaluated using univariate and multivariate Cox regression analyses to identify the factors associated with treatment outcomes (11). Covariates were considered statistically significant at $P < 0.05$. Statistical analyses were performed using the R statistical software (version 4.1.1).

Results

Demographic and clinical characteristics of study patients

A total of 262 patients pathologically diagnosed with pelvic and genitourinary RMS were obtained from the SEER database.

Abbreviations: RMS, Rhabdomyosarcoma; RT, radiotherapy; SEER, The Surveillance, Epidemiology, and End Results Program; LASSO, least absolute shrinkage and selection operator; OS, overall survival; CSS, cancer-specific survival; C-index, concordance index; AUC, receiver operating characteristic curve; PSM, propensity score matching.

The eligibility criteria and demographic characteristics are shown in Figure 1 and Table 1. Pelvic and genitourinary RMS were more common in men (79.4%) than in women (20.6%). The testis was the most common primary site (46.6%, n=122), followed by the urinary bladder (21.8%, n=57) and prostate (18.3%, n = 48), and the uterus and other sites made up the remaining 13.3%. Embryons were the most common histological subtype (79.7%, 209), followed by alveolar (11.1%, 29), spindle, and other (9.2%) subtypes. Most patients (96.6%) received chemotherapy, 77.5% were treated with cancer-directed surgery, and nearly half of the patients received RT. The tumor stage was categorized as localized, regional, or distant. Localized tumors (43.9%, 115) included single or multifocal invasive tumors confined to the primary site or in but not beyond the capsule. Regions (30.9%, 81) included direct extension into peripheral tissues, such as blood vessels. Distant (25.2%, 66) included metastasis and invasion of distant lymph nodes, bones, etc.

Feature selection and prognostic signature building

In total, nine variables (sex, age, race, site, histology, summary stage, chemotherapy, cancer-directed surgery, and RT) were included in the analysis. According to the LASSO Cox regression analysis results, eight variables (sex, age, site, histology, summary stage, chemotherapy, cancer-directed surgery, and RT) were identified as potential risk factors for OS and CSS (Figure 2).

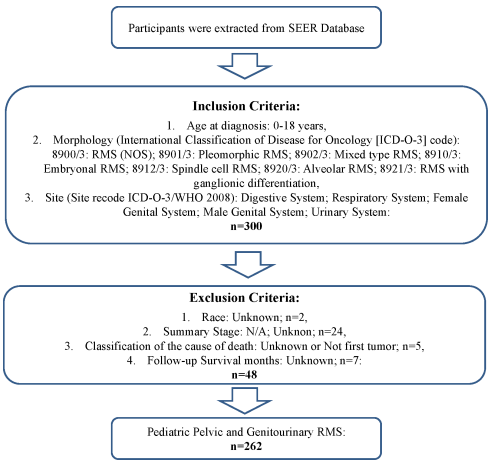


FIGURE 1
Flowchart of inclusion and exclusion criteria for patient selection.

Identification of independent prognostic factors

A multivariable Cox regression model was used to search for OS- and CSS-related prognostic factors (Table 2). According to multivariable Cox analyses, age, site, summary stage, histology, cancer-directed surgery, and chemotherapy were significantly associated with OS ($P < 0.05$), while age, site, summary stage, and cancer-directed surgery were significantly associated with CSS ($P < 0.05$). These variables were defined as the independent prognostic factors for OS and CSS.

Nomogram construction and validation

As shown in Figures 3, 4, we constructed nomograms by incorporating prognostic factors to predict the 1- and 5-year OS and CSS. The predicted nomogram showed excellent consistency with actual survival outcomes. The accuracy of the nomogram was evaluated using the C-index and AUC values of the ROC. The C-index for OS nomogram was 85.13% (95% CI: 83.06%-87.20%) and for CSS nomogram was 86.45% (95% CI: 82.43%-86.91%). The calibration curve revealed substantial concordance between the actual observation and prediction (Figure 5). The AUC values of the 1- and 5-year OS/CSS were 0.892/0.887, and 0.873/0.857, respectively (Figure 6). These results indicate that the nomograms showed excellent predictive performance and calibration.

Survival analysis of different prognostic factors

Survival time data were analyzed for each variable, and the median follow-up was 5.1 years. Kaplan-Meier survival curves were constructed, and the median OS (half of the time of death) was calculated for each variable.

The overall survival and cancer-specific survival curves are illustrated in Figures 7, 8, respectively. There was a significant difference between the different age cohorts, and the patients in the 15-19 age group had the poorest OS ($P < 0.05$) (Figure 7A), and CSS ($P < 0.05$) (Figure 8A), which is consistent with the results of the multivariate Cox regression analysis (Table 2). Comparing the OS and CSS of patients with different organs involved RMS (Figures 7B, 8B), the uterus, urinary bladder, and testis had better prognostic survival than prostate-involved RMS ($P < 0.001$). In terms of different stages (Figures 7C, 8C), localized-stage RMS showed the best OS and CSS, while distant-stage RMS had the poorest prognostic survival (median OS, 28 months; median CSS, 29 months). There was a significant prognostic difference between the alveolar group and other sub-histological groups (embryonal, etc.) ($P < 0.005$) (Figures 7D, 8D). In the univariate survival analysis of the RT

10.3389/fonc.2022.992738

TABLE 1 Patient characteristics.

	Alveolar RMS (N = 29)	Embryona lRMS (N = 209)	ganglionic differentiation RMS (N = 2)	Mixed RMS (N = 8)	Pleomorphic RMS (N = 2)	Spindle RMS (N = 12)	Overall (N = 262)
Sex							
Female	8 (27.6%)	43 (20.6%)	1 (50.0%)	0 (0%)	1 (50%)	1 (8.3%)	54 (20.6%)
Male	21 (72.4%)	166 (79.4%)	1 (50.0%)	8 (100%)	1 (50.0%)	11 (91.7%)	208 (79.4%)
Age							
0-4	8 (27.6%)	87 (41.6%)	1 (50.0%)	4 (50.0%)	0 (0%)	6 (50.0%)	106 (40.5%)
5-9	3 (10.3%)	39 (18.7%)	1 (50.0%)	1 (12.5%)	0 (0%)	2 (16.7%)	46 (17.6%)
10-14	5 (17.2%)	35 (16.7%)	0 (0%)	1 (12.5%)	0 (0%)	1 (8.3%)	42 (16.0%)
15-19	13 (44.8%)	48 (23.0%)	0 (0%)	2 (25.0%)	2 (100%)	3 (25.0%)	68 (26.0%)
Race							
Black	8 (27.6%)	40 (19.1%)	0 (0%)	1 (12.5%)	0 (0%)	3 (25.0%)	52 (19.8%)
Other	1 (3.4%)	11 (5.3%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	13 (5.0%)
White	20 (69.0%)	155 (74.2%)	2 (100%)	7 (87.5%)	2 (100%)	8 (66.7%)	194 (74.0%)
Unknown	0 (0%)	3 (1.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1.1%)
Site							
Anus, Anal Canal and Anorectum	1 (3.4%)	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	0 (0%)	2 (0.8%)
Kidney and Renal Pelvis	1 (3.4%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (0.8%)
Prostate	11 (37.9%)	37 (17.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	48 (18.3%)
Rectum	1 (3.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.4%)
Testis	8 (27.6%)	94 (45.0%)	1 (50.0%)	7 (87.5%)	1 (50.0%)	11 (91.7%)	122 (46.6%)
Urinary Bladder	2 (6.9%)	55 (26.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	57 (21.8%)
Vulva	5 (17.2%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	7 (2.7%)
Ovary	0 (0%)	2 (1.0%)	1 (50.0%)	0 (0%)	1 (50.0%)	0 (0%)	4 (1.5%)
Uterus	0 (0%)	19 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	19 (7.3%)
Summary stage							
Distant	15 (51.7%)	47 (22.5%)	0 (0%)	2 (25.0%)	1 (50.0%)	1 (8.3%)	66 (25.2%)
Localized	5 (17.2%)	100 (47.8%)	1 (50.0%)	2 (25.0%)	0 (0%)	7 (58.3%)	115 (43.9%)
Regional	9 (31.0%)	62 (29.7%)	1 (50.0%)	4 (50.0%)	1 (50.0%)	4 (33.3%)	81 (30.9%)
Cancer-directed surgery							
No (1)	11 (37.9%)	41 (19.6%)	1 (50.0%)	0 (0%)	0 (0%)	1 (8.3%)	54 (20.6%)
Recommended but not performed (2)	1 (3.4%)	4 (1.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (1.9%)
Yes	17 (58.6%)	164 (78.5%)	1 (50.0%)	8 (100%)	2 (100%)	11 (91.7%)	203 (77.5%)

(Continued)

TABLE 1 Continued

	Alveolar RMS (N = 29)	Embryona lRMS (N = 209)	ganglionic differentiation RMS (N = 2)	Mixed RMS (N = 8)	Pleomorphic RMS (N = 2)	Spindle RMS (N = 12)	Overall (N = 262)
Radiation recode							
Only after surgery	12 (41.4%)	62 (29.7%)	0 (0%)	4 (50.0%)	2 (100%)	3 (25.0%)	83 (31.7%)
None	8 (27.6%)	106 (50.7%)	2 (100%)	4 (50.0%)	0 (0%)	8 (66.7%)	128 (48.9%)
Radiation (without surgery)	9 (31.0%)	36 (17.2%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	46 (17.6%)
before and After surgery	0 (0%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.4%)
Intraoperative radiation	0 (0%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.4%)
Prior to surgery	0 (0%)	3 (1.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1.1%)
Chemotherapy							
Yes	29 (100%)	200 (95.7%)	2 (100%)	8 (100%)	2 (100%)	12 (100%)	253 (96.6%)
No	0 (0%)	9 (4.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9 (3.4%)

(1) No (surgery): cancer-directed surgery was not performed because it was not recommended by physician due to patient risk factors;
(2) Recommended but not performed: cancer-directed surgery was recommended by the patient's physician but was not performed as part of the therapy because it was refused by the patients or patients' guardian or some other reason.

vs. the no-RT group, the RT group had poorer survival than the no-RT group (Figures 7E, 8E). Patients with alveolar RMS had the poorest OS and CSS among the assessed histology groups (median OS, 36.2 months). Interestingly, patients who underwent surgery had significantly better prognostic survival ($P < 0.001$) (Figure 7F, Figure 8F). There was no significant difference in prognostic survival between the different races ($P>0.05$) (Appendix Figures 1A, 2A) and sexes ($P>0.05$) (Appendix Figures 1. B and 2. B).

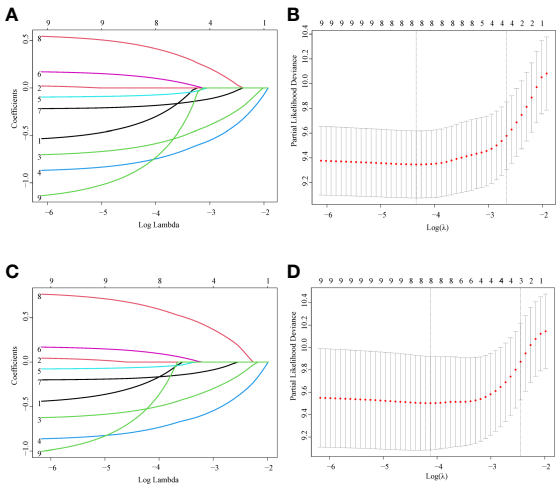


FIGURE 2
Feature selection using LASSO Cox regression. **(A)** OS: The binomial deviance is plotted versus log (λ). **(B)** OS: LASSO coefficient profiles of the eight clinical features. A coefficient profile plot is produced versus the log (λ). **(C)** CSS: The binomial deviance is plotted versus log (λ). **(D)** CSS: LASSO coefficient profiles of the eight clinical features. A coefficient profile plot is produced versus the log (λ).

On subtype analyses of different therapeutic regimens (Appendix Tables A1-A3, Figure 9), cancer-directed surgery was associated with improved OS, while treatment with RT in combination with chemotherapy or surgery failed to provide a survival benefit ($P > 0.05$). Interestingly, the significant survival difference between different therapeutic regimens was only observed in patients with distant metastasis, which may be explained by that the majority of patients treated at an early stage of disease can have satisfactory outcomes, while there are higher requirements for therapy options considering the survival of patients with metastasis. The effect of RT on the survival of patients with pelvic and genitourinary RMS requires further investigation.

Survival outcomes after propensity score analysis

A propensity score analysis was performed to match 55 patients who received RT and 55 who did not receive RT (Table 3). As a result, characteristics such as age (P =1), site (P =0.628), and histology (P =0.58) were balanced, without significant differences.

TABLE 2 Multivariable Cox regression analysis of predictors of OS and CSS for pelvic and genitourinary RMS.

Risk factor	Overall Survival		Cancer-Specific Survival	
	HR (1) (95% CI)	*P*	HR (1) (95% CI)	*P*
external beam radiotherapy				
Yes	0.947 (0.488 - 1.839)	0.872	1.145 (0.556- 2.358)	0.713
No		Reference		
Cancer-directed surgery				
Yes	1.404 (0.643- 2.923)	0.365	1.405 (0.643- 3.069)	0.394
Recommended but not performed	3.382 (1.072- 10.670)	**0.038**	3.973 (1.231- 12.825)	**0.021**
No		Reference		
Chemotherapy				
Yes	0.221 (0.056, 0.878)	**0.032**	0.270 (0.053, 1.380)	0.116
No		Reference		
Histology Subtype				
Embryonal RMS	1.312 (0.622- 2.765)	0.475	1.194 (0.547- 2.606)	0.657
Others (2)	3.356 (1.016, 11.087)	**0.047**	3.213 (0.949, 10.879)	0.061
Alveolar		Reference		
Summary Stage				
Localized	0.065 (0.022- 0.192)	**<0.001**	0.070 (0.022- 0.219)	**<0.001**
Regional	0.271 (0.140- 0.523)	**<0.001**	0.277 (0.138- 0.555)	**<0.001**
Distant		Reference		
Site				
Prostate	1.225 (0.352, 4.270)	0.750	1.107 (0.300, 4.081)	0.878
Testis	0.150 (0.039, 0.584)	**0.006**	0.165 (0.040, 0.682)	**0.013**
Urinary Bladder	0.429 (0.132, 1.388)	0.158	0.468 (0.137, 1.599)	0.226
Uterus	0.090 (0.435, 0.822)	**0.0329**	–	–
Others (3)		Reference		
Age				
10-14	2.019 (0.793- 5.142)	0.141	1.577 (0.570- 4.362)	0.381
15-19	2.984 (1.442- 6.175)	**0.003**	2.370 (1.257- 5.863)	**0.011**
5-9	1.088 (0.447- 2.724)	0.857	1.130 (0.447- 2.856)	0.795
0-4		Reference		
Gender				
Male	0.577 (0.216, 1.539)	0.356	0.625 (0.231, 1.695)	0.356
Female		Reference		

(1) HR, Hazard ratio.
(2) Others: Ganglionic differentiation RMS; Mixed RMS; Pleomorphic RMS; Spindle RMS.
(3) Others: Anus, Anal Canal and Anorectum; Kidney and Renal Pelvis; Rectum; Vulva; Ovary.
Bold values means P value <;0.05.

Kaplan Meier plot (Figure 10) revealed approximately 75% OS at 5 years of follow-up for both the RT and no-RT groups (P =0.773) and yielded a statistically insignificant univariable HRs of 1.113 (0.537–2.307, P =0.773). The 5-year CSS was approximately 75% for both the RT and no-RT groups (P =0.49), with a statistically insignificant univariable HRs of 1.320 (0.599–2.91, P =0.49). In multivariable Cox regression analysis (Table 4), the implementation of RT was associated with worse prognostic survival, but no statistically significant changes were observed in 5-year survival (OS: HR=1.431; 95% CI: 0.661–3.099; P =0.364; CSS: HR=1.601; 95% CI: 0.700–3.662; P =0.265).

Discussion

RMS lesions in the pelvic cavity and urogenital system are often close to large blood vessels and vital organs and usually grow too large to be completely removed before diagnosis. According to the Children's Oncology Group (COG) and groups in Europe (the Soft Tissue Sarcoma Committee of the COG, etc.) (12), multimodal therapy combining surgical resection, preoperative/postoperative chemotherapy, and RT has been the overall treatment philosophy. However, there are no guidelines, especially for pediatric pelvic and genitourinary RMS. Therefore, the prognostic factors for pediatric pelvic and

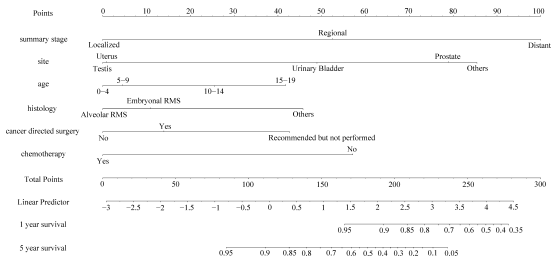


FIGURE 3
The nomogram of predicting OS of patients aged 0-19 years with pelvic and genitourinary RMS.

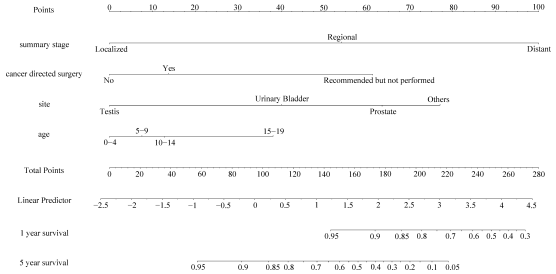


FIGURE 4
The nomogram of predicting CSS of patients aged 0-19 years with pelvic and genitourinary RMS.

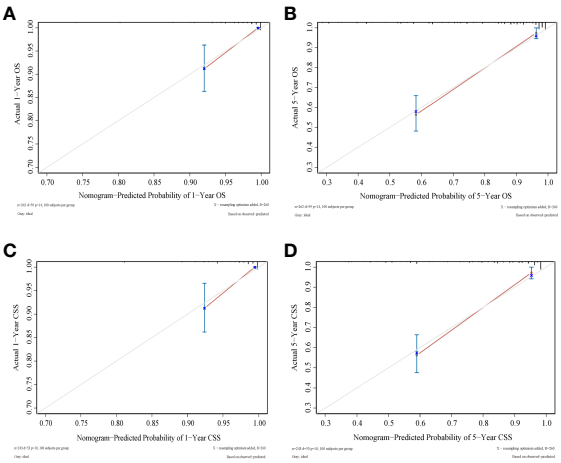


FIGURE 5
Calibration curves to predict **(A)** 1-year OS; **(B)** 5-year OS; **(C)** 1-year CSS; **(D)** 1-year CSS. Predicted survival is plotted on the x-axis, and actual survival is plotted on the *y*-axis.

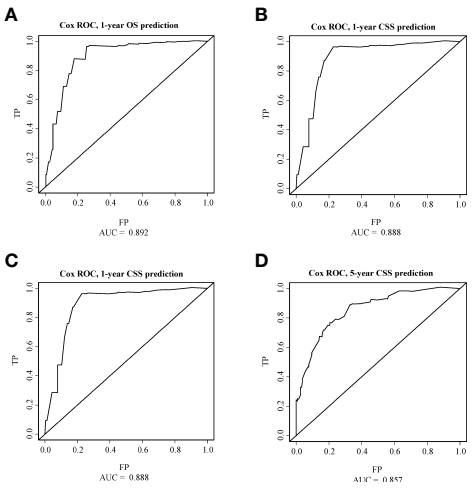


FIGURE 6
ROC curves of OS and CSS. **(A)** The ROC curve of 1-year OS; **(B)** The ROC curve of 5-year OS; **(C)** The ROC curve of 1-year CSS; **(D)** The ROC curve of 5-year CSS.

genitourinary RMS and the outcomes of different treatments are worth exploring. To the best of our knowledge, this is the first population-based study to determine prognostic factors and assess the outcomes of pediatric patients with pelvic and genitourinary RMS.

This study found that the histological subtype, age, pathological stage, and site were significantly associated with OS and CSS. According to the histological features, RMS can be divided into two main subtypes (embryonal and alveolar) and other rare subtypes (pleomorphic, anaplastic, etc.). In this study, alveolar RMS showed a poorer prognosis than embryonal, pleomorphic, and anaplastic RMS, which is consistent with the recent understanding of pediatric RMS (13–15). It is more difficult to treat alveolar RMS than other subtypes (embryonal type, etc.) due to its discrete location, metastatic tendency and

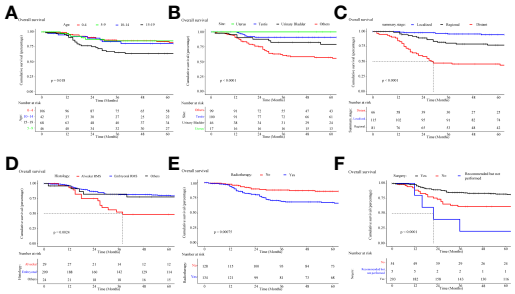


FIGURE 7
Kaplan-Meier curve of overall survival stratified by **(A)** age, **(B)** site, **(C)** stage, **(D)** histology, **(E)** radiotherapy, **(F)** cancer-directed surgery.

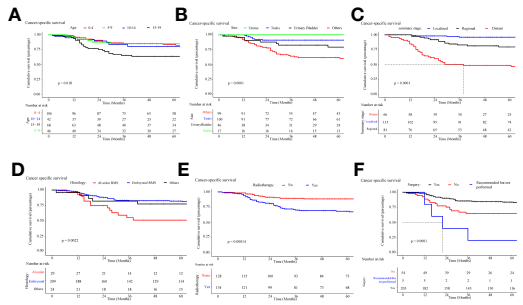


FIGURE 8
Kaplan-Meier curve of cancer-specific survival stratified by **(A)** age, **(B)** site, **(C)** stage, **(D)** histology, **(E)** radiotherapy, **(F)** cancer-directed surgery.

high degree of malignancy. Therefore, in this study, the proportion of patients with alveolar type who received surgery was higher than that of patients with embryonal type. For different age groups, patients in the 15-19 group showed a significantly worse prognostic survival than patients aged 0-15 years (OS: HR 2.984, 95% CI 1.442-6.175, $P < 0.01$**; CSS: HR 2.370, 95% CI 1.257-5.863, $P < 0.05$*). The same report as shown by the Italian and German Soft Tissue Cooperative Groups that age <10 years at diagnosis and embryonal histology are favorable prognostic factors (16, 17). The prognosis of pediatric metastatic RMS remains poor (18). We found that a higher tumor stage was associated with worse prognosis (OS: localized stage: HR 0.070, 95% CI 0.022-0.192, $P < 0.001$; CSS: localized stage: HR 0.065, 95% CI 0.022-0.219, $P < 0.001$). We also found that RMS in the testis and uterus has a much better prognosis than RMS in other locations, suggesting that pediatric RMS in reproductive organs may has better prognostic survival.

For decades, surgery associated with chemotherapy and RT has been the gold standard treatment for patients with RMS (19). Our results support the idea that surgery is the most important therapy in RMS treatment (20), as we found that cancer-directed surgery significantly improved five-year OS/CSS. In our subgroup analysis, chemotherapy improved the survival rate (HR=0.221; 95% CI, 0.056–0.878; P=0.032). Although some

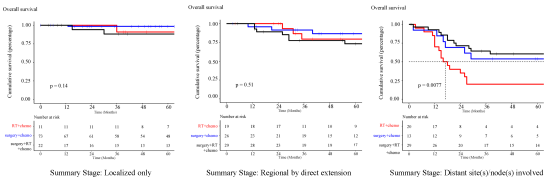


FIGURE 9
Kaplan-Meier curve of survival stratified by different therapeutic regimens.

studies have shown different results in that preoperative and/or postoperative chemotherapy is ineffective (21, 22), chemotherapy continues to be recommended due to surgical benefits (tumor shrinkage after chemotherapy). Collectively, appropriate chemotherapy can confer overall prognostic survival for patients with pelvic and genitourinary RMS.

To the best of our knowledge, this analysis included the largest cohort of children with pelvic and genitourinary RMS treated with RT. The propensity score analysis showed that postoperative RT provided no significant survival benefit for children with pelvic and genitourinary RMS. Similar results were obtained in a study that included 237 patients with vaginal/uterine RMS, and the pooled analysis showed no statistical difference (P>0.05) in OS between patients with and without RT (10-year OS: 94% without RT *vs.* 89% with RT) (23). In subgroup Cox analysis, our study also showed that RT failed to provide survival benefits, even with chemotherapy or surgery. Although the clinical efficacy of RT remains to be evaluated, American clinical guidelines for RMS in children still recommend RT as a standard treatment. Approximately 75% of children with RMS are treated with RT, and long-term side effects have frequently been observed at different sites (4). When pelvic radiation is used for pediatric RMS, RT-related toxicity can affect normal tissues, which may result in growth asymmetries, cystitis, infertility, and sexual dysfunction (24–26). Late radiation-induced toxicity also includes decreased bone growth, increased risk of secondary malignancy, and hematuria (27–29). Therefore, we must be aware of the potential toxicity to patients' lives (25). Currently, at least three randomized clinical trials of pediatric RMS to evaluate the survival impact of RT are in progress (NCT00002995, NCT01626170, and NCT00075582). As data from NCT00002995 have shown, no evidence suggests that reduced RT dose has a negative impact on 5-year failure-free survival (FFS) and OS (localized, stage1/2/3 embryonal RMS, treated with surgical resection and chemotherapy (VA/VAC)) (30). For patients with localized RMS of the vagina, RT-related long-term effects are sometimes unacceptable, especially in children under 24 months of age (31).

Current studies concentrating on the prognostic factors of RMS were mostly based on pathological factors at the time of initial diagnosis, which did not calculate the dynamic changes that occur during the disease process (32). Our study is the first to predict the prognostic survival of pelvic and genitourinary RMS throughout the disease course. We defined different risk factors and constructed relevant nomograms to predict the OS/CSS in patients with pelvic and genitourinary RMS. These nomograms may help predict prognosis more accurately.

Our study has several limitations. Since pediatric RMS is a rare type of pediatric cancer, and the incidence of different subtypes varies greatly (33, 34), there are certain different subtype proportions in this study. The conclusions about alveolar and other rare subtypes need to be validated in more cases in future. Given the retrospective nature of this study, all

TABLE 3 Characteristics of patients after propensity score matching.

	None (N = 55)	Yes (N = 55)	P.value
Sex			
Female	11 (20.0%)	9 (16.4%)	0.805
Male	44 (80.0%)	46 (83.6%)	
age			
0-4	27 (49.1%)	27 (49.1%)	1
5-9	9 (16.4%)	9 (16.4%)	
10-14	7 (12.7%)	8 (14.5%)	
15-19	12 (21.8%)	11 (20.0%)	
race			
Black	12 (21.8%)	11 (20.0%)	0.883
Other	3 (5.5%)	2 (3.6%)	
White	40 (72.7%)	42 (76.4%)	
site			
Others	9 (16.4%)	12 (21.8%)	0.628
Tesis+Uterus+Urinary Bladder	46 (83.6%)	43 (78.2%)	
histology			
Alveolar RMS etc.	9 (16.4%)	6 (10.9%)	0.58
Embryonal RMS	46 (83.6%)	49 (89.1%)	
summary.stage			
Distant	16 (29.1%)	16 (29.1%)	0.789
Localized	19 (34.5%)	22 (40.0%)	
Regional	20 (36.4%)	17 (30.9%)	
chemotherapy			
No	0 (0%)	0 (0%)	1
Yes	55 (100%)	55 (100%)	
cancer.directed.surgery			
No	6 (10.9%)	6 (10.9%)	1
Yes	49 (89.1%)	49 (89.1%)	

TABLE 4 Cox regression analysis of the patients after propensity score matching.

Univariate Regression Analysis (OS)			
Risk factor	HR (95% CI)		*P* Value
RT (Yes)	1.113 (0.537- 2.307)		0.773
RT (No)		Ref	
Univariate Regression Analysis (CSS)			
RT (Yes)	1.320 (0.599- 2.91)		0.49
RT (No)		Ref	
Multivariate Cox Proportional Hazards Analysis (OS)			
RT (Yes)	1.431 (0.661, 3.099)		0.364
RT (No)		Ref	
Multivariate Cox Proportional Hazards Analysis (CSS)			
RT (Yes)	1.601 (0.699, 3.662)		0.265
RT (No)		Ref	

Signif. codes: '***' 0.001; '**' 0.01; '*' 0.05, '.' 0.1; ' ' 1.

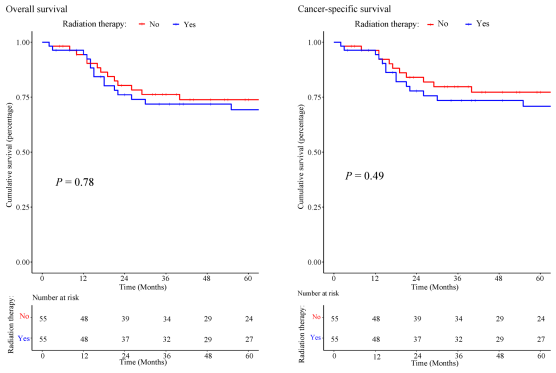


FIGURE 10
Kaplan-Meier of overall survival and cancer-specific survival in patients treated with or without RT.

analyses were subject to selection biases and imbalances in unquantified variables. Of particular importance, specific regimens and dosages for chemotherapy and RT were unavailable. We used several analytical approaches to address potential unmeasured confounding factors, including LASSO regression, multivariable adjustment, and propensity score analysis. All the analytical approaches provided generally consistent results.

In conclusion, age at diagnosis of < 15 years, non-alveolar histological subtype, early tumor stage (localized/regional), specific sites (uterus and testis), and treatment (cancer-directed surgery and chemotherapy) were favorable prognostic factors. The survival nomogram is a user-friendly tool composed of readily available baseline objective data elements that allow robust estimates of survival in patients, overcoming the epistemic uncertainty of the prognostication of this disease. The results of this analysis suggest that RT may not associated with improved prognostic survival in patients with pelvic and genitourinary RMS. Randomized trials to evaluate the impact of RT in pediatric pelvic and genitourinary RMS are warranted. In contrast, cancer-directed surgery can significantly extend life expectancy and increase the cure rate, and chemotherapy may play a role as an adjuvant therapy to improve the curative effects.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

JWu and XS designed the research and wrote the paper, JC and JM provided professional advice, and JWu performed the research and analyzed the data. All authors read and commented on the paper. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by Open Foundation of Key Laboratory of Digital Technology in Medical Diagnostics of Zhejiang Province (Grant No. SZZD202217), National Natural Science Foundation of China (Grant No. 81573516).

Acknowledgments

We thank Dr. Robert M. Dorazio for providing support in research design and analytical process.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.992738/full#supplementary-material

References

1. Bergeron C, Jenney M, De Corti F, Gallego S, Merks H, Glosli H, et al. Embryonal rhabdomyosarcoma completely resected at diagnosis: The European paediatric soft tissue sarcoma study group RMS2005 experience. *Eur J Cancer* (2021) 146:21–9. doi: 10.1016/j.ejca.2020.12.025

2. Shapiro DD, Harel M, Ferrer F, McKenna PH. Focusing on organ preservation and function: Paradigm shifts in the treatment of pediatric genitourinary rhabdomyosarcoma. *Int Urol Nephrol* (2016) 48(7):1009–13. doi: 10.1007/s11255-016-1285-0

3. Althumairi AA, Efron JE. Genitourinary considerations in reoperative and complex colorectal surgery. *Clin Colon Rectal Surg* (2016) 29(2):145–51. doi: 10.1055/s-0036-1580629

4. Punyko JA, Mertens AC, Gurney JG, Yasui Y, Donaldson SS, Rodeberg DA, et al. Long-term medical effects of childhood and adolescent rhabdomyosarcoma: A report from the childhood cancer survivor study. *Pediatr Blood Cancer* (2005) 44 (7):643–53. doi: 10.1002/pbc.20310

5. Heyn R, Raney RB Jr., Hays DM, Tefft M, Gehan E, Webber B, et al. Late effects of therapy in patients with paratesticular rhabdomyosarcoma. intergroup rhabdomyosarcoma study committee. *J Clin Oncol* (1992) 10(4):614–23. doi: 10.1200/JCO.1992.10.4.614

6. Tinkle CL, Pappo A, Wu J, Mao S, Hua CH, Shulkin BL, et al. Efficacy and safety of limited-margin conformal radiation therapy for pediatric rhabdomyosarcoma: Long-term results of a phase 2 study. *Int J Radiat Oncol Biol Phys* (2020) 107(1):172–80. doi: 10.1016/j.ijrobp.2020.01.011

7. Doll KM, Rademaker A, Sosa JA. Practical guide to surgical data sets: Surveillance, epidemiology, and end results (SEER) database. *JAMA surg.* (2018) 153(6):588–9. doi: 10.1001/jamasurg.2018.0501

8. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials* (1996) 17(4):343–6. doi: 10.1016/0197-2456(96) 00075-X

9. FE HJ. rms: Regression modeling strategies. In: *R package version 5.1-4*. https://CRAN.R-project.org/package=rms. Accessed 22 Jan 2020

10. Austin PC. Optimal caliper widths for propensity-score matching when estimating differences in means and differences in proportions in observational studies. *Pharm Stat* (2011) 10(2):150–61. doi: 10.1002/pst.433

11. George B, Seals S, Aban I. Survival analysis and regression models. *J Nucl Cardiol* (2014) 21(4):686–94. doi: 10.1007/s12350-014-9908-2

12. Stevens MC, Rey A, Bouvet N, Ellershaw C, Flamant F, Habrand JL, et al. Treatment of nonmetastatic rhabdomyosarcoma in childhood and adolescence: third study of the international society of paediatric oncology–SIOP malignant mesenchymal tumor 89. *J Clin Oncol* (2005) 23(12):2618–28. doi: 10.1200/JCO.2005.08.130

13. Gartrell J, Pappo A. Recent advances in understanding and managing pediatric rhabdomyosarcoma. *F1000* (2020) 9:F1000. doi: 10.12688/f1000research.22451.1

14. Louie RR, Harris M, Shields K. Survival with persistent metastatic alveolar rhabdomyosarcoma. *J Oncol Pract* (2017) 13(5):341–2. doi: 10.1200/JOP.2016.018788

15. Eguía-Aguilar P, López-Martínez B, Retana-Contreras C, Perezpeña-Diazconti M. Alveolar rhabdomyosarcoma: Origin and prognostic implications of molecular findings. *Boletin medico del Hosp Infantil Mexico* (2016) 73(6):405–10. doi: 10.1016/j.bmhimx.2016.09.001

16. Cecchetto G, Bisogno G, Treuner J, Ferrari A, Mattke A, Casanova M, et al. Role of surgery for nonmetastatic abdominal rhabdomyosarcomas: A report from the Italian and German soft tissue cooperative groups studies. *Cancer* (2003) 97 (8):1974–80. doi: 10.1002/cncr.11285

17. Raney RB, Stoner JA, Walterhouse DO, Andrassy RJ, Donaldson SS, Laurie F, et al. Results of treatment of fifty-six patients with localized retroperitoneal and pelvic rhabdomyosarcoma: A report from the intergroup rhabdomyosarcoma study-IV, 1991-1997. *Pediatr Blood Cancer* (2004) 42(7):618–25. doi: 10.1002/pbc.20012

18. Oberlin O, Rey A, Lyden E, Bisogno G, Stevens MC, Meyer WH, et al. Prognostic factors in metastatic rhabdomyosarcomas: Results of a pooled analysis from united states and European cooperative groups. *J Clin Oncol* (2008) 26 (14):2384–9. doi: 10.1200/JCO.2007.14.7207

19. Egas-Bejar D, Huh WW. Rhabdomyosarcoma in adolescent and young adult patients: Current perspectives. *Adolesc health Med Ther* (2014) 5:115–25. doi: 10.2147/AHMT.S44582

20. Smolle MA, Szkandera J, Andreou D, Palmerini E, Bergovec M, Leithner A. Treatment options in unresectable soft tissue and bone sarcoma of the extremities and pelvis - a systematic literature review. *EFORT Open Rev* (2020) 5(11):799–814. doi: 10.1302/2058-5241.5.200069

21. Hiraga H, Ozaki T. Adjuvant and neoadjuvant chemotherapy for osteosarcoma: JCOG bone and soft tissue tumor study group. *Jpn J Clin Oncol* (2021) 51(10):1493–7. doi: 10.1093/jjco/hyab120

22. Woll PJ, Reichardt P, Le Cesne A, Bonvalot S, Azzarelli A, Hoekstra HJ, et al. Adjuvant chemotherapy with doxorubicin, ifosfamide, and lenograstim for resected soft-tissue sarcoma (EORTC 62931): A multicentre randomised controlled trial. *Lancet Oncol* (2012) 13(10):1045–54. doi: 10.1016/S1470-2045 (12)70346-7

23. Minard-Colin V, Walterhouse D, Bisogno G, Martelli H, Anderson J, Rodeberg DA, et al. Localized vaginal/uterine rhabdomyosarcoma-results of a pooled analysis from four international cooperative groups. *Pediatr Blood Cancer* (2018) 65(9):e27096. doi: 10.1002/pbc.27096

24. Raney B, Anderson J, Jenney M, Arndt C, Brecht I, Carli M, et al. Late effects in 164 patients with rhabdomyosarcoma of the bladder/prostate region: A report from the international workshop. *J Urol* (2006) 176(5):2190–4. doi: 10.1016/j.juro.2006.07.064

25. Blitzer GC, Bradley KA. Four ways to decrease late toxicity from pelvic radiation therapy in children and young adults. *Pract Radiat Oncol* (2021) 11 (6):434–40. doi: 10.1016/j.prro.2021.07.001

26. Lockney NA, Friedman DN, Wexler LH, Sklar CA, Casey DL, Wolden SL. Late toxicities of intensity-modulated radiation therapy for head and neck rhabdomyosarcoma. *Pediatr Blood Cancer* (2016) 63(9):1608–14. doi: 10.1002/pbc.26061

27. Heyn R, Haeberlen V, Newton WA, Ragab AH, Raney RB, Tefft M, et al. Second malignant neoplasms in children treated for rhabdomyosarcoma. Intergroup rhabdomyosarcoma study committee. *J Clin Oncol* (1993) 11(2):262–70. doi: 10.1200/JCO.1993.11.2.262

28. Krasin MJ, Xiong X, Wu S, Merchant TE. The effects of external beam irradiation on the growth of flat bones in children: Modeling a dose-volume effect. *Int J Radiat Oncol Biol Phys* (2005) 62(5):1458–63. doi: 10.1016/j.ijrobp.2005.01.024

29. Cotter SE, Herrup DA, Friedmann A, Macdonald SM, Pieretti RV, Robinson G, et al. Proton radiotherapy for pediatric bladder/prostate rhabdomyosarcoma: Clinical outcomes and dosimetry compared to intensity-modulated radiation therapy. *Int J Radiat Oncol Biol Phys* (2011) 81(5):1367–73. doi: 10.1016/j.ijrobp.2010.07.1989

30. Raney RB, Walterhouse DO, Meza JL, Andrassy RJ, Breneman JC, Crist WM, et al. Results of the intergroup rhabdomyosarcoma study group D9602 protocol, using vincristine and dactinomycin with or without cyclophosphamide and radiation therapy, for newly diagnosed patients with low-risk embryonal rhabdomyosarcoma: A report from the soft tissue sarcoma committee of the children's oncology group. *J Clin Oncol* (2011) 29(10):1312–8. doi: 10.1200/JCO.2010.30.4469

31. Walterhouse DO, Meza JL, Breneman JC, Donaldson SS, Hayes-Jordan A, Pappo AS, et al. Local control and outcome in children with localized vaginal rhabdomyosarcoma: A report from the soft tissue sarcoma committee of the children's oncology group. *Pediatr Blood Cancer* (2011) 57(1):76–83. doi: 10.1002/pbc.22928

32. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J Cancer* (2018) 103:356–87. doi: 10.1016/j.ejca.2018.07.005

33. . Available at: https://www.cancer.gov/types/soft-tissue-sarcoma/hp/rhabdomyosarcoma-treatment-pdq.

34. Sultan I, Qaddoumi I, Yaser S, Rodriguez-Galindo C, Ferrari A. Comparing adult and pediatric rhabdomyosarcoma in the surveillance, epidemiology and end results program, 1973 to 2005: an analysis of 2,600 patients. *J Clin Oncol* (2009) 27 (20):3391–7. doi: 10.1200/JCO.2008.19.7483

10.3389/fonc.2022.992738

Appendixes

APPENDIX TABLE A1 Survival analyses of different therapeutic regimens-localized.

Regimen	Overall Survival		
	HR	95% CI	*P*
Surgery+chemo	0.177	0.011 to 2.823	0.220
Surgery+ RT+chemo	1.357	0.123 to 14.970	0.803
RT+chemo	Reference	Reference	

APPENDIX TABLE A2 Survival analyses of different therapeutic regimens-regional.

Regimen	Overall Survival		
	HR	95% CI	*P*
Surgery+chemo	0.706	0.143 to 3.503	0.671
Surgery+ RT+chemo	1.514	0.391 to 5.862	0.548
RT+chemo	Reference	Reference	

APPENDIX TABLE A3 Survival analyses of different therapeutic regimens-distant.

Regimen	Overall Survival		
	HR	95% CI	*P*
Surgery+chemo	0.444	0.182 to 1.087	0.077.
Surgery+ RT+chemo	0.323	0.151 to 0.690	0.004 **
RT+chemo	Reference	Reference	

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 24 November 2022
DOI 10.3389/fonc.2022.1019911

OPEN ACCESS

EDITED BY
Jilong Yang,
Tianjin Medical University Cancer Institute and Hospital, China

REVIEWED BY
Kenji Nakano,
Cancer Institute Hospital of Japanese Foundation for Cancer Research, Japan
Lu Xie,
Peking University People's Hospital, China

*CORRESPONDENCE
Jiayong Liu
liujiayong_doc@163.com

†These authors have contributed equally to this work and share first authorship

SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 15 August 2022
ACCEPTED 08 November 2022
PUBLISHED 24 November 2022

CITATION
Tan Z, Liu J, Xue R, Fan Z, Bai C, Li S, Gao T, Zhang L and Wang X (2022) Clinical features and therapeutic outcomes of alveolar soft part sarcoma in children: A single-center, retrospective study.
Front. Oncol. 12:1019911.
doi: 10.3389/fonc.2022.1019911

Clinical features and therapeutic outcomes of alveolar soft part sarcoma in children: A single-center, retrospective study

Zhichao Tan†, Jiayong Liu*†, Ruifeng Xue†, Zhengfu Fan, Chujie Bai, Shu Li, Tian Gao, Lu Zhang and Xinyu Wang

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Bone and Soft Tissue Tumor, Peking University Cancer Hospital and Institute, Beijing, China

Background: Alveolar soft part sarcoma (ASPS) is a rare sarcoma that has been shown to be highly effective to antiangiogenic agents and immune checkpoint inhibitors, but most reported studies about ASPS were concentrated on adult population. In this study, we aimed to describe the clinical features and therapeutic outcomes of ASPS in children.

Methods: We retrospectively reviewed the records of patients with ASPS in our institution since Jan 2015. All patients included in this study were pathologically confirmed ASPS and aged under 12 years at the time of initial diagnosis. Demographic characteristics, tumor sizes, primary tumor sites, metastasis, treatments used, therapeutic responses and survivals were evaluated.

Results: We identified a total of 56 patients to be initially diagnosed as ASPS since Jan 2015. A predisposition of high occurrence in head and neck (32.1%) was observed (versus 41.1% in limbs and 21.4% in trunk). 26 (46.4%) patients developed metastasis at the time of diagnosis or during follow-up. Tumors in tongue, pharynx and larynx had the least likelihood to metastasize (7.7%, $P<0.05$). Observation was recommended for 15 stage IV patients with only pulmonary metastasis. 7 (46.7%) patients remained stable until last follow up. The 1-year PFS rate was 83.3% and median progression-free survival time (PFS) was 29.4 months. 15 patients with progressive disease received mono or combined therapy. 11 patients received PD-1 monotherapy. 2 patients achieved partial response and 5 stable disease. The overall response rate was 18.2%. The median PFS of this group was 22.0 months, and the 1-year PFS rate was 70.0%. 4 patients received a combination therapy of PD-1 inhibitors plus tyrosine kinase inhibitors. All of them remained stable. No disease-related death occurred during follow-up.

Conclusions: ASPS exhibits a higher occurrence in head and neck in children. ASPS originating from glossopharyngeal region tends to have a lower metastasis rate. ASPS displays a more indolent growth pattern in children, which makes observation a preferable choice for children with sole

pulmonary metastasis. Pediatric ASPS appears to be less effective to targeted therapy and immunotherapy than adults. The treatment of progressive ASPS in children remains challenging.

KEYWORDS

alveolar soft part sarcoma, pediatric, clinical features, targeted therapy, immunotherapy

Introduction

Alveolar soft part sarcoma (ASPS) is a rare histologic subtype of soft tissue sarcomas, which represents less than 1% of all soft tissue sarcomas (1). It is characterized by the frequent presence of the chromosomal rearrangement der (2)t(X;17)(p11;q25), leading to a chimeric APSCR1-TFE3 transcription factor (3). About one third of ASPS occur in children and adolescents, accounting for about 4.5% of all soft tissue sarcomas in this population (4). The majority of our knowledge regarding to ASPS comes from the adult population, and the size of the studies is relatively small. The largest study so far, which used the National Cancer Database, identified 293 ASPS patients aged 18 and over, and concluded that ASPS present with a high rate of metastasis (59%) and an indolent course when compared to other malignant soft tissue tumors (5). Resection remains the standard treatment when feasible. For stage IV ASPS, traditional cytotoxic chemotherapy enacted poor activity (overall response rate (ORR) less than 10%) (6). However, the recent progress in targeted and immune therapy had led to delighting results in ASPS. A recent meta-analysis evaluating the efficacy of immune checkpoint inhibitors in sarcomas reported an ORR of 35% in the ASPS subgroup (7). Tyrosine kinase inhibitors, such as cediranib and pazopanib, achieved an ORR of 20-35% (8, 9). Recently, a phase II study of TQB2450 (a PD-L1 antibody) in combination with anlotinib, achieved an ORR of 75.0% in the ASPS subgroup (10).

Pediatric series are very limited due to the extreme rarity of ASPS in children. The largest study consisted of 51 children and adolescents with ASPS from seven European Cooperative Groups (11). Another study, consisting of 69 patients, included patients younger than 30 years with ASPS from four major cancer centers in North America (12). So we conducted this retrospective, single-center study to better identify the clinical features and outcomes of ASPS in children,.

Materials and methods

Patient data

The clinical data of children (under 12 years old) diagnosed with ASPS since January, 2015 was retrospectively collected from our institution. The study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained for all patients. All patients included in this study were pathologically confirmed ASPS. Case records were analyzed for anatomical site of primary disease and metastasis, extent of surgery, adjuvant radiation, types and duration of medical therapy, response to therapy, time to progression or recurrence, and follow-up. The TNM postsurgical staging was employed.

Therapeutic strategy

If possible, a wide excision of primary tumor would be recommended for all patients. For those with unresectable primary tumor or can be only resected with positive surgical margin (such as tumors located at orbit), radical radiotherapy would be conducted. After eradication of primary tumor, patients without metastasis and patients with only pulmonary metastasis were suggested to closely observe. We would start medical intervention for patients with extra-pulmonary metastasis, and those who were confirmed to have progressive disease during follow up. Tyrosine kinase inhibitors (TKIs) monotherapy, PD-1 inhibitors monotherapy, or a combination of these two agents would be employed according to the recommendation of the attending physician and the choice of the patient's family. The PD-1 inhibitors we employed included pembrolizumab, torpalimab and sintilimab; the TKIs included anlotinib and pazopanib. We generally used 1/3-1/2 standard dose of these drugs, and the specific dose would be adjusted according to the age and weight of the patient. This therapeutic strategy was discussed and approved by the ethics committee of Peking University Cancer Hospital.

Statistical analysis

Standard descriptive statistics were used. The means were compared by using independent sample t-test. Survival was analyzed using the Kaplan-Meier method. In the metastasis-free group, event-free survival (EFS) was defined as the time from the date of diagnosis to date of metastasize, relapse or last follow-up. In IV stage patients, for the observation group, progression-free survival (PFS) was defined as the time from

the diagnosis of metastasis to the date of progression or last follow-up; for the treatment group, PFS was defined as the start of medical treatment to the date of progression or last follow-up. Overall survival (OS) was defined as time from the date of diagnosis to death or last follow-up. The monitor of primary tumor site and metastasis was conducted by using computed tomography (CT) or magnetic resonance imaging (MRI). For patients with measurable disease (tumor diameter greater or equal to 1cm), we used the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) to evaluate the response to medical treatment. For patients without evaluable tumors (tumor diameter less than 1cm), we defined complete response (CR) as the all visible tumor disappeared with no residual disease, stable disease (SD) as tumor increased less than 5mm or decreased but still visible, progressive disease (PD) as the tumor increased more than 5mm. Significance (P<0.05, two-tailed) was determined by log-rank test with respect to EFS, PFS and OS, and by Cox regression models for the univariate and multivariate analysis for the following covariates: patient age, gender, primary tumor site, primary tumor size, medical treatment. Statistical analysis was completed using SPSS 25.0.

Results

A total of 56 children under 12 years old were identified since 2015. The clinical characteristics of them are shown in Table 1. A predisposition of female dominance was observed (64.3%). The primary tumor was located mostly in limb (41.1%), head and neck (31.1%), and trunk (21.4%). Nearly half of the patients (46.4%) had metastasis at diagnosis or during follow-up. Different origins of tumor demonstrated different metastatic rate. Tumors located in tongue, pharynx and larynx were least likely to metastasize (7.7%, versus limb 65.2%, trunk 41.7%, abdominal and pelvic and retroperitoneal cavity, 66.7%, orbit 75%, P<0.05)(Figure 1). Interestingly, the diameter of tumor originating from this area (tongue, pharynx and larynx) was not significantly different from other parts (limb: 4.24cm, trunk: 3.42cm, abdominal and pelvic and retroperitoneal cavity: 4.6cm; orbit: 2.2cm; tongue, pharynx and larynx: 3.09cm, P>0.1). Gender and age were neither prognostic factor for metastasis.

48 patients were metastasis-free when diagnosed, and 20 (41.7%) had metastasis during follow-up. The median event-free survival was 63.2 months (Figure 2A). 15 patients had only pulmonary metastasis, including those who were discovered when diagnosed or during follow-up. These patients were recommended to receive no medical treatment and observation. In this observation group, 7 patients (46.7%) remained stable during follow-up. The median PFS was 29.4 months, and the 1-year PFS rate was 83.3% (Figure 2B).

15 patients received medical treatment, including 9 patients with extra-pulmonary metastasis and 6 patients with progression of pulmonary metastasis during follow-up. No patient received TKIs monotherapy and 11 patients received PD-1 inhibitor monotherapy as initial treatment. 2 of 11 (18.2%) had PR, 6 had SD and 3 had PD. The median PFS for monotherapy group was 22.0 months, and the 1-year PFS rate was 70.0% (Figure 2C). 4 patients received TKIs and PD-1 inhibitors combination therapy. All of them remained SD. No CR were recorded in either monotherapy or combination therapy group. No disease-related death was reported during follow-up, thus the median OS was not reached.

Discussion

As a rare cancer, it is hard to evaluate the biological behavior and therapeutic outcomes of ASPS with large-scale clinical trials, especially in pediatric population. Only several studies concentrated on pediatric and adolescent ASPS patients (11–13). However, there are some limitations in these studies. Firstly, both targeted therapy and immunotherapy have achieved good results in ASPS in recent years, and have changed the outcomes of many patients. Therefore, the results of these emerging therapies and the survival data of patients need to be updated. Secondly, these studies do not further distinguish between pediatric and adolescent populations. More data are needed to

TABLE 1 Characteristics of patients.

	Number (%)
Gender	
Male	20 (35.7)
Female	36(64.3)
Age (median, year)	6.5(1-12)
Primary tumor site	
Limb	23 (41.1)
Trunk	12 (21.4)
Abdominal, pelvic, retroperitoneal cavity	3 (5.4)
Head and neck	18 (32.1)
Tongue, pharynx and larynx	13 (23.2)
Orbit	4 (7.1)
others	1 (1.8)
Primary tumor diameter (median, cm)	3.6 (0.6-9.0)
T stage	
1	35 (62.5)
2	10 (17.9)
NA	11 (19.6)
N stage	
N0	54 (96.5)
N+	2 (3.5)
Metastasis	
No	30 (53.6)
Yes	26 (46.4)
Follow-up time (median, month)	34.7 (3.1-84.5)

NA, not available.

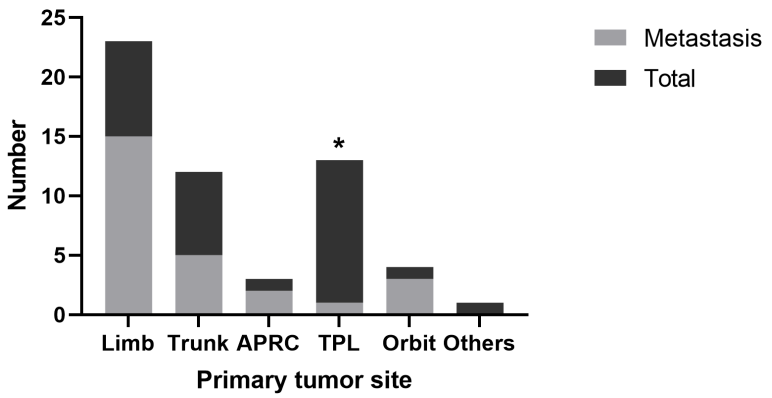


FIGURE 1
Tumor metastasis from different origin. APRC, abdominal, pelvic and retroperitoneal cavity; TPL, tongue, pharynx and larynx.

confirm the differences in clinical features, prognosis, and treatment response between ASPS in children and adolescents. Our study was aimed to evaluate clinical features of ASPS in the specific population: children under 12 years old. 56 patients were included in this study. As far as we know, this is the largest ASPS pediatric population reported to date.

We identified a relatively higher rate of occurrence and a lower rate of metastasis relating to a particular primary tumor site in head and neck, especially in glossopharyngeal region. In the adult ASPS population, tumors originating from head and neck only consisted of 3.4% (5); in the children and adolescent ASPS population, the ratio was 15%-19% (11, 12) while in our study it was 32.1%. On the contrary to its high occurrence, the metastatic rate of glossopharyngeal region was surprisingly low (7.7%). We are not the first to discover this phenomenon since Hagerty et al. also reported ASPS from head and neck had a lesser metastatic rate (40%) compared to extremity origins (73%) (5). Those researchers attributed it to the higher visibility of lesions arising from head and neck, which leads to an earlier medical intervention, but we doubt this theory because in our result, the diameter of tumors from this region was not significantly smaller than other origins, although the small sample size limited the validity. Tumor biology might be a contributing factor. Previous studies regarding head and neck sarcomas mostly reported distant metastatic rate around 20%. In 3 retrospective reviews examining a total of 403 patients with head and neck sarcomas, the authors reported a distant metastatic rate of 17.6%, with a total of 71 metastasis (14–16). Notably, Torosian et al. reported 565 patients with soft tissue sarcomas, in which 18% (52/237) of patients with extremity sarcomas and 19% (4/21) of patients with head and neck sarcomas had metastatic disease. This result was contradictory to the early intervention theory. We would like to suppose that head and neck should not be dealt as a whole when analyzing risk factors for metastasis. In our result, ASPS from head and neck had a metastatic rate of 23.5%, but tumors from glossopharyngeal region displayed a much lower distant metastatic rate compared to orbit origin (1/13 vs 3/4).

The 5-year survival rate of stage IV ASPS patients was approximately 50%-60% in recent research (5, 17). Thirty years ago, a same rate of 5-year survival rate was observed with localized disease (2). The data implies that the treatment has improved in recent years. But it still remains unclear when to start intervention with medical treatment because of its relatively indolent growth. In our result, about half of the patients with only pulmonary metastasis remained SD without any intervention, and the 1-year PFS rate reached up to 83.3%. We also noticed that there were some cases with only pulmonary metastasis remained a long period of stability during follow-up in the reports of other investigators (12). Moreover, there is further evidence that ASPS plays a more indolent course in young patients. Lieberman et al. reported that the frequency of metastasis at presentation increased with the age at diagnosis, and the median survival decreased with it: patients at age interval 0-9 years had 17% metastatic rate and median survival of 20 years, while patients at age over 30 years had 32% metastatic rate and median survival of only 5 years (2). Therefore, we would like to imply that observation can be the first choice for stage IV ASPS children with only pulmonary metastasis.

For patients with disease progression or extra-pulmonary metastasis, TKIs and/or PD-1 inhibitors were recommended by some authoritative institutions, such as the National Comprehensive Cancer Network (NCCN). In our study, no patient chose TKIs monotherapy and most patients chose PD1 inhibitors as the initial treatment, because they were concerned about the adverse effects and the rebound effects after drug resistance of TKIs. In adults, previous clinical trials demonstrated that PD-1 monotherapy achieved an ORR of 25%-37.2% (18, 19). However, only 16.7% of children with ASPS in our study responded to PD-1 inhibitors. 27.2% (3/11) patients had disease progression during monotherapy, which was

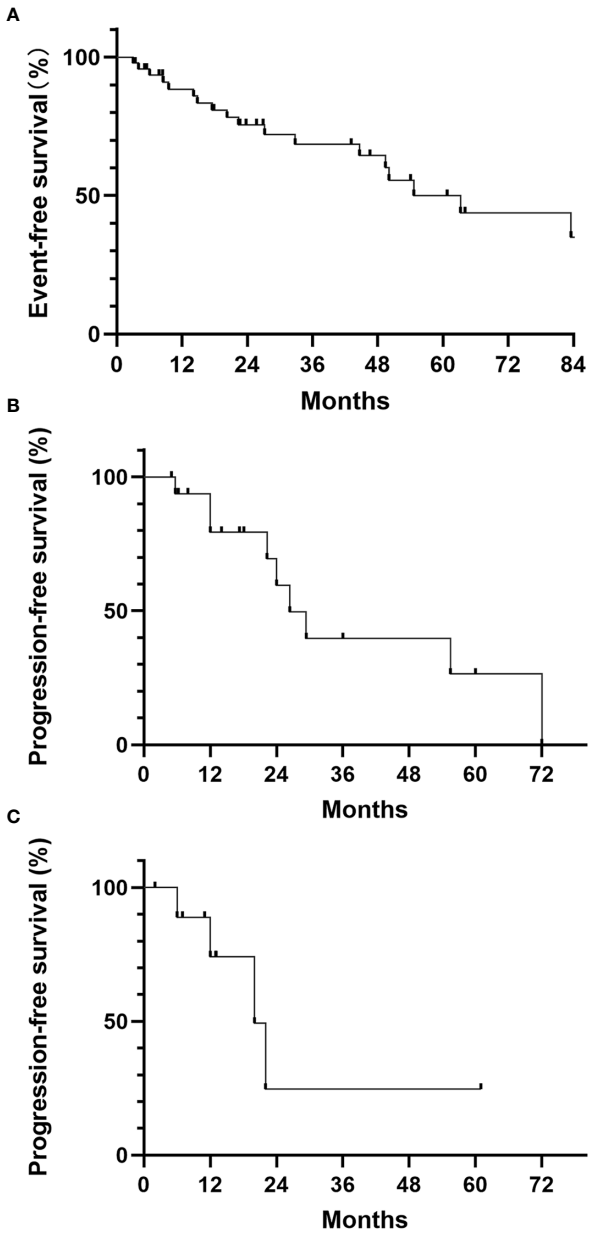


FIGURE 2
(A) Event-free survival of localized ASPS; **(B)** Progression-free survival for the observation group; **(C)** Progression-free survival for the PD-1 monotherapy group.

higher compared to adult population (<10%) (18, 19). For combination therapy, all 4 patients who received TKIs and PD-1 inhibitors stayed SD. This was a disappointing result because in clinical trials recruiting adults, combination therapy achieved an ORR of 58.3%-75.0% (10, 20). These findings may imply that although ASPS in children is more indolent, but it is less sensitive to target and immune therapies. In the field of immunosenescence, there is a theory that tumors in the elderly have more mutations than the young, because of a long period of exposure to environment and/or intrinsic mutagens, which contributes more neoantigens to be targeted by T cells of the host immune system. This theory might be a potential explanation to our finding (21). But historic data about pediatric ASPS is very limited, and previous clinical trials excluded patients under 18 years old, therefore, we may need more clinical reviews to confirm the validity of this hypothesis.

There are some limitations in our study. First, due to the long-time span and technological reasons, not all patients had the

testing of ASPL-TFE3 fusion gene. To make the pathologic confirmation, all histologic slides were reviewed by experienced pathologists and the necessary immunohistochemistry was performed, including TFE3. Second, selection bias was inevitable as a single center, retrospective study. We collected records of every confirmed ASPS patients to reduce the bias. Moreover, the imaging assessment was confirmed by both the clinician and the radiologist. Third, the number of patients receiving combination therapy was very limited, which undermines the validity of our results. Considering its rarity, further cooperation of large centers is essential to make high-level clinical evidence.

Conclusion

ASPS affects head and neck more often in children. Head and neck should be more subdivided when analyzing risk factors, in which tumors originating from glossopharyngeal region had the lowest metastatic rate. The mechanism needs further exploration. ASPS displays a more indolent growth pattern in children than adults. For children with sole pulmonary metastasis, observation might be a preferrable choice. As for progressive disease, pediatric ASPS appears to be more resistant to both PD-1 inhibitors and TKIs than adults. Therefore, it is still challenging to find an effective treatment for children with ASPS.

Data availability statement

The dataset included private data of patients. Requests to access the datasets should be directed to ZT, alveolarpku@126.com.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Peking University Cancer Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ZT, JL, and RX contributed equally to this work. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LX declared a shared parent affiliation with the authors to the handling editor at the time of review.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Folpe AL, Deyrup AT. Alveolar soft-part sarcoma: a review and update. *J Clin Pathol* (2006) 59(11):1127–32. doi: 10.1136/jcp.2005.031120

2. Lieberman PH, Brennan MF, Kimmel M, Erlandson RA, Garin-Chesa P, Flehinger BY. Alveolar soft-part sarcoma. a clinico-pathologic study of half a century. *Cancer* (1989) 63(1):1–13. doi: 10.1002/1097-0142(19890101)63:1<1::AID-CNCR2820630102>3.0.CO;2-E

3. Hodge JC, Pearce KE, Wang X, Wiktor AE, Oliveira AM, Greipp PT. Molecular cytogenetic analysis for TFE3 rearrangement in Xp11.2 renal cell carcinoma and alveolar soft part sarcoma: validation and clinical experience with 75 cases. *Mod Pathol* (2014) 27(1):113–27.

4. Ferrari A, Casanova M, Collini P, Meazza C, Luksch R, Massimino M, et al. Adult-type soft tissue sarcomas in pediatric-age patients: experience at the istituto nazionale tumori in Milan. *J Clin Oncol* (2005) 23(18):4021–30. doi: 10.1200/JCO.2005.02.053

5. Hagerty BL, Aversa J, Diggs LP, Dominguez DA, Ayabe RI, Blakely AM, et al. Characterization of alveolar soft part sarcoma using a large national database. *Surgery* (2020) 168(5):825–30. doi: 10.1016/j.surg.2020.06.007

6. Reichardt P, Lindner T, Pink D, Thuss-Patience PC, Kretzschmar A, Dörken B. Chemotherapy in alveolar soft part sarcomas. what do we know? *Eur J Cancer* (2003) 39(11):1511–6. doi: 10.1016/S0959-8049(03)00264-8

7. Saerens M, Brusselaers N, Rottey S, Decruyenaere A, Creytens D, Lapeire L. Immune checkpoint inhibitors in treatment of soft-tissue sarcoma: A systematic review and meta-analysis. *Eur J Cancer* (2021) 152:165–82. doi: 10.1016/j.ejca.2021.04.034

8. Judson I, Morden JP, Kilburn L, Leahy M, Benson C, Bhadri V, et al. Cediranib in patients with alveolar soft-part sarcoma (CASPS): a double-blind, placebo-controlled, randomised, phase 2 trial. *Lancet Oncol* (2019) 20(7):1023–34. doi: 10.1016/S1470-2045(19)30215-3

9. Urakawa H, Kawai A, Goto T, Hiraga H, Ozaki T, Tsuchiya H, et al. Phase II trial of pazopanib in patients with metastatic or unresectable chemoresistant sarcomas: A Japanese musculoskeletal oncology group study. *Cancer Sci* (2020) 111(9):3303–12. doi: 10.1111/cas.14542

10. Liu J, Gao T, Tan Z, Li S, Xu J, Bai C, et al. Phase II study of TQB2450, a novel PD-L1 antibody, in combination with anlotinib in patients with locally advanced or metastatic soft tissue sarcoma. *Clin Cancer Res* (2022) 28(16):3473–9. doi: 10.1158/1078-0432.CCR-22-0871

11. Orbach D, Brennan B, Casanova M, Bergeron C, Mosseri V, Francotte N, et al. Paediatric and adolescent alveolar soft part sarcoma: A joint series from European cooperative groups. *Pediatr Blood Cancer* (2013) 60(11):1826–32. doi: 10.1002/pbc.24683

12. Flores RJ, Harrison DJ, Federman NC, Furman WL, Huh WW, Broaddus EG, et al. Alveolar soft part sarcoma in children and young adults: A report of 69 cases. *Pediatr Blood Cancer* (2018) 65(5):e26953. doi: 10.1002/pbc.26953

13. Casanova M, Ferrari A, Bisogno G, Cecchetto G, Basso E, De Bernardi B, et al. Alveolar soft part sarcoma in children and adolescents: A report from the soft-tissue sarcoma Italian cooperative group. *Ann Oncol* (2000) 11(11):1445–9. doi: 10.1023/A:1026579623136

14. Mattavelli D, Miceli R, Radaelli S, Mattavelli F, Cantù G, Barisella M, et al. Head and neck soft tissue sarcomas: prognostic factors and outcome in a series of patients treated at a single institution. *Ann Oncol* (2013) 24(8):2181–9. doi: 10.1093/annonc/mdt126

15. Dudhat SB, Mistry RC, Varughese T, Fakih AR, Chinoy RF. Prognostic factors in head and neck soft tissue sarcomas. *Cancer* (2000) 89(4):868–72. doi: 10.1002/1097-0142(20000815)89:4<868::AID-CNCR20>3.0.CO;2-9

16. Tran LM, Mark R, Meier R, Calcaterra TC, Parker RG. Sarcomas of the head and neck. prognostic factors and treatment strategies. *Cancer* (1992) 70 (1):169–77.

17. Paoluzzi L, Maki RG. Diagnosis, prognosis, and treatment of alveolar soft-part sarcoma: A review. *JAMA Oncol* (2019) 5(2):254–60. doi: 10.1001/jamaoncol.2018.4490

18. Naqash AR, O'Sullivan Coyne GH, Moore N, Sharon E, Takebe N, Fino KK, et al. Phase II study of atezolizumab in advanced alveolar soft part sarcoma (ASPS). *J Clin Oncol* (2021) 39(15_suppl):11519–. doi: 10.1200/JCO.2021.39.15_suppl.11519

19. Yang J, Dong L, Yang S, Han X, Han Y, Jiang S, et al. Safety and clinical efficacy of toripalimab, a PD-1 mAb, in patients with advanced or recurrent malignancies in a phase I study. *Eur J Cancer* (2020) 130:182–92. doi: 10.1016/j.ejca.2020.01.028

20. Wilky BA, Trucco MM, Subhawong TK, Florou V, Park W, Kwon D, et al. Axitinib plus pembrolizumab in patients with advanced sarcomas including alveolar soft-part sarcoma: A single-centre, single-arm, phase 2 trial. *Lancet Oncol* (2019) 20(6):837–48. doi: 10.1016/S1470-2045(19)30153-6

21. Pawelec G. Does patient age influence anti-cancer immunity? *Semin Immunopathol* (2019) 41(1):125–31. doi: 10.1007/s00281-018-0697-6

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 16 February 2023
DOI 10.3389/fonc.2023.1000949

OPEN ACCESS

EDITED BY
Jilong Yang,
Tianjin Medical University Cancer Institute and Hospital, China

REVIEWED BY
Changye Zou,
The First Affiliated Hospital of Sun Yat-sen University, China
Hongru Shen,
Tianjin Medical University Cancer Institute and Hospital, China

*CORRESPONDENCE
Jin Deng
gydengjin@yeah.net
Nannan Zhao
znneye66@hainmc.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 22 July 2022
ACCEPTED 27 January 2023
PUBLISHED 16 February 2023

CITATION
Qu G, Xu Y, Qu Y, Qiu J, Chen G, Zhao N and Deng J (2023) Identification and validation of a novel ubiquitination-related gene *UBE2T* in Ewing's sarcoma. *Front. Oncol.* 13:1000949. doi: 10.3389/fonc.2023.1000949

Identification and validation of a novel ubiquitination-related gene *UBE2T* in Ewing's sarcoma

Guoxin Qu[1,2,3†], Yuanchun Xu[3,4†], Ye Qu[4†], Jinchao Qiu[2], Guosheng Chen[2], Nannan Zhao[1,5*] and Jin Deng[2*]

[1]Department of Orthopaedics, The First Affiliated Hospital of Hainan Medical University, Hainan Medical University, Haikou, China, [2]Department of Emergency, The Affiliated Hospital of Guizhou Medical University, Guizhou Medical University, Guiyang, China, [3]Department of General Surgery, State Key Laboratory of Trauma, Burns and Combined Injury, Research Institute of Surgery, Daping Hospital, Army Medical University, Chongqing, China, [4]Department of Trauma Surgery, The Second Affiliated Hospital of Hainan Medical University, Hainan Medical University, Haikou, China, [5]Department of Ophthalmology, The First Affiliated Hospital of Hainan Medical University, Hainan Medical University, Haikou, China

Background: Ewing's sarcoma (ES) is one of the most prevalent malignant bone tumors worldwide. However, the molecular mechanisms of the genes and signaling pathways of ES are still not well sufficiently comprehended. To identify candidate genes involved in the development and progression of ES, the study screened for key genes and biological pathways related to ES using bioinformatics methods.

Methods: The GSE45544 and GSE17618 microarray datasets were downloaded from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were identified, and functional enrichment analysis was performed. A protein–protein interaction (PPI) network was built, and key module analysis was performed using STRING and Cytoscape. A core-gene was gained and was validated by the validation dataset GSE67886 and immunohistochemistry (IHC). The diagnostic value and prognosis evaluation of ES were executed using, respectively, the ROC approach and Cox Regression.

Results: A total of 187 DEGs, consisting of 56 downregulated genes and 131 upregulated genes, were identified by comparing the tumor samples to normal samples. The enriched functions and pathways of the DEGs, including cell division, mitotic nuclear division, cell proliferation, cell cycle, oocyte meiosis, and progesterone-mediated oocyte maturation, were analyzed. There were 149 nodes and 1246 edges in the PPI network, and 15 hub genes were identified according to the degree levels. The core gene (*UBE2T*) showed high expression in ES, validated by using GSE67886 and IHC. The ROC analysis revealed *UBE2T* had outstanding diagnostic value in ES (AUC = 0.75 in the training set, AUC = 0.90 in the validation set). Kaplan-Meier (analysis of survival rate) and Cox Regression analyses indicated that UBE2T was a sign of adverse results for sufferers with ES.

Conlusion: *UBE2T* was a significant value biomarker for diagnosis and treatment of ES, thereby presenting a novel potential therapeutic target for ES as well as a new perspective for assessing the effect of treatment and prognostic prediction.

KEYWORDS

***UBE2T*, ubiquitination, Ewing's sarcoma, prognosis, diagnosis, biomarker**

Highlights

1. Ewing's sarcoma (ES)-related DGEs were verified ground on the GEO database and TCGA database.
2. In all 187 DEGs and 15 hub genes were closely associated with the progression of ES.
3. One key alteration gene (*UBE2T*) was differently expressed between tumor specimens and normal specimens, suggesting that this gene may be a latent prognosis predictor for ES stufferers.
4. Validation set and IHC confirmed that the UBE2T was overexpressed in ES but not in normal tissues.
5. In patients with ES, *UBE2T* can be used as a biomarker with important diagnostic value as well as an independent prognosis. The discovery of *UBE2T* will provide a new perspective for ES research.

1 Introduction

Ewing sarcoma (ES), an invasive ossature and soft-tissue cancer, is a frequent malignant bone tumor, ranking second among the pediatric population, and it also affects adolescents (1, 2). Presently, the standard of treatment for ES involves multimodal therapy, including surgical resection, local radiation therapy, and intensive multiagent chemotherapy (3). Despite tremendous advances in diagnosis, treatment, and prognosis of this illness with the advancement of medicine, nonspecific clinical features of ES give rise to symptoms that are unremarkable in the early stages, and high metastasis and recurrence rates have become the main poor outcomes of treatment(2, 4, 5). Furthermore, as the complete mechanisms of the molecular pathology for ES tumorigenesis and progression are unknown, there are few efficacious ways available to early diagnose the disease, resulting in a high mortality rate and death rate. As a result, successfully implementing diagnosis and treatment approaches requires a thorough insight into the mechanisms of the molecular biology underlying tumorigenesis, multiplication, and recurrence of ES.

Affymetrix techniques and bioinformatics research have been increasingly employed to monitor gene expression levels in recent decades, allowing for the efficiently identification of DEGs and functional pathways related to the tumorigenesis and development of ES. There are many microarray data sets shared and kept in accessible web databases. In order to the screening of additional molecular markers, many microarray data information for identifying ES genes can be available from the database. To evaluate DEGs between tumor specimens and nontumor specimens, two microarray datasets collected from the GEO (Gene Expression Omnibus) (6) data bank were obtained and processed in this study. And to research the latent functions of these DEGs, we applied GO (Gene Ontology) (7, 8), KEGG (Kyoto Encyclopedia of Genes and Genomes) (9) pathway enrichment study, and PPI (protein–protein interaction) network research. Finally, the current investigation discovered a total of 187 DEGs, 15 hub genes and 1core DEGs, and further validation experiments, diagnostic value and prognosis analysis were carried out on core-DEGs, which discovered a valuable latent biomarker for the diagnosis, remedy, and prognosis evaluation of ES (Figure 1).

2 Materials and methods

2.1 Microarray data

This currently study obtained two training datasets, the GSE17618 (10) and GSE45544 (11) from the GEO data bank (http://www.ncbi.nlm.nih.gov/geo) (6), which is a publicly accessible functional genetic and genomic data repository for high-throughput gene expression information, chips, and microarrays. The GSE45544 dataset (including 20 ES and 22 noncancerous tissue specimens) is dependent on Affymetrix GPL6244 platform data (Affymetrix Human Gene 1.0 ST Array), whereas the GSE17618 dataset (73 specimens, ES n = 55 and normal n = 18) is built on Affymetrix GPL570 platform data (Affymetrix Human Genome U133 Plus 2.0 Array). Furthermore, the GSE68776 (12) from the GPL570 platform (Affymetrix Human Exon 1.0 ST Array) was extracted as a validation dataset (ES specimens n = 32; normal n = 33) to be used later.

2.2 Identification of DEGs

The GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) was executed to pick out the DEGs between ES and noncancerous

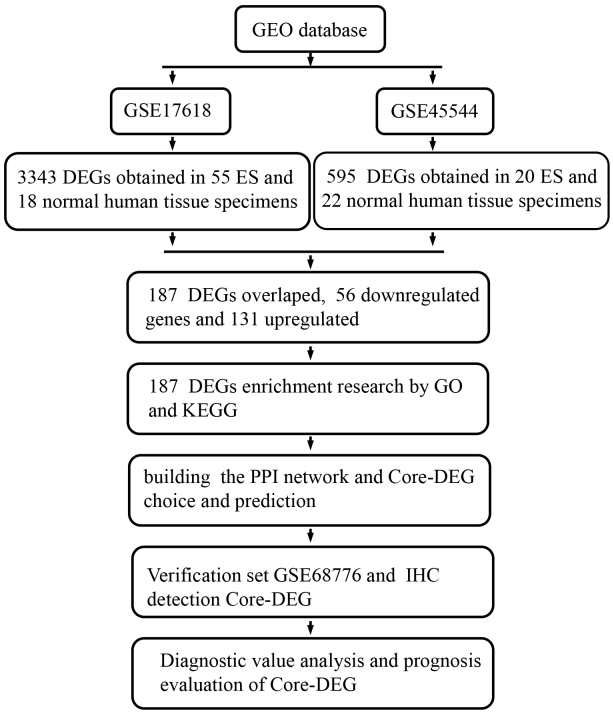


FIGURE 1
This study's flow diagram. GEO, Gene Expression Omnibus database; ES, Ewing's sarcoma; DEGs, Differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; IHC, Immunohistochemical.

specimens. GEO2R is a web-based interactive tool that allows clients to gain data by comparing two or more GEO series datasets to discover DEGs from experimental results, to analyze DEGs, and to determine highly expressed and negatively regulated DEGs between ES samples and normal specimens. And, the adjusted P values (adj. P) and Benjamini and Hochberg false discovering rates were employed to provide a balance between the excavation of statistically meaningful genes and the restrictions of false-positives. Probe sets with no associated gene symbols were eliminated, as were genes with multiple probe sets. |logFC (fold change)| ≥ 2 and adj. P values < 0.01 were deemed statistically meaningful.

2.3 DEG enrichment research using GO and KEGG

The Databank for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.ncifcrf.gov) (6.8 version)(13) is a publicly viewable laboratory biological information data bank that incorporates analytical and statistical tools based on biological analysis and offers a wide range with a suite of integrated functional annotation data of proteins and genes to continue investigating biological data information. GO is a computer-based bioinformatics software that is mostly used to annotate genes and research their biological processes (7). KEGG is a computer statistical resource database that evaluates high-standard biological processes and function systems from a wide range of molecular datasets and discovers pathways in which DEGs may play a major role (14). The DAVID online information system was implemented for the functional study of DEG biology. $P < 0.05$ was accepted as statistical significance.

2.4 Building and analyzing of the PPI network and module

The PPI network was built utilizing the STRING (Search Tool for the Retrieval of Interacting Genes, http://string-db.org) (11.0 version) (15) online database. Assessing and analyzing protein-protein interactions may critically reveal the mechanisms of the generation or progression of illnesses. An interaction with a combined score > 0.4 was considered statistically significant. Cytoscape (3.8.2 version) is an available open-source bioinformatics software tool utilized to visualize network systems of molecular interaction (16). And, the MCODE (Molecular Complex Detection) (2.0 version) plugin of Cytoscape is an application (APP) software used to search densely connected regions in large PPI networks (17) and to verify the most major module section (MCODE-DEGs). the following conditions for filtering were used: MCODE scores are greater than 5, the degree cutoff is 2, the node score cutoff is 0.2, the maximum depth is 100, and the k-score is 2,. and the biological process investigation was carried out with Cytoscape ClueGO (18) (version 2.5.8). Next, a hierarchical clustering (using R the pheatmap package) of MCODE-DEGs was implemented based on the expression profiling of training datasets.

2.5 Core-DEG choice and verification set detection

The degree levels in the cytoHubba (19) Cytoscape plugin were implemented to define the hub genes. Cytoscape ClueGO (18) (version 2.5.8) was used to depict the biological process investigation of core genes. Furthermore, mutant survival, including overall survival and illness-free survival, was assessed to further screen the hub gene core-DEG employing Kaplan-Meier methods in the cBioPortal web tool (http://www.cbioportal.Org) (20). Then, GSE68776 (ES n = 32, control n = 33) was used to validate the expression of the core-DEG, which was depicted in the volcano plot by the "ggplot2" software.

2.6 Immunohistochemistry experiment

A total of 11 paraffin-embedded Ewing's sarcoma tissues (8 males (72.73%) and 3 females (27.27%)) were provided by Daping Hospital (Chongqing, China). All patients signed a written informed consent form. 3mm tumor paraffin sections were blocked for 1 hour at room temperature with sheep serum blocking solution (Zhongshan Jinqiao, China), then diluted 1/100 with anti-UBE2T antibody and anti-CD99 antibody(Cohesion Biosciences, UK) at 4 °C overnight. Then, for 2 hours at room temperature, goat anti rabbit secondary antibody (1:200 dilution; Biyuntian, China) was administered for color development (Zhongshan Jinqiao, China), and the nucleus was stained with hematoxylin. The results were then examined under an optical microscope (Ningbo Konfoong, China). Besides, to assess the area and density of stained regions, as well as the internal grating optical density (IOD) values of IHC sections, Image Pro Plus version 6.0 software (Media Cybernetics, Rockville, MD, USA) was employed. The signal density of a tissue region chosen at random from five locations was counted and statistically assessed using a blind approach.

2.7 Diagnostic value analysis of *UBE2T* in the ES

The receiver operating characteristic curve (ROC) technique in the Python package was executed to analyze core gene diagnostic effectiveness according to the training set and validation set.

2.8 Identification of DEGs subgroups in ES

To better understand the biological phenotype of MCODE-DEGs regulation in the tissue of ES patients, the MCODE-DEGs based on gene expression profiles in the training dataset were grouped using Consensus Cluster Plus (21). UMAP (version 0.2.7.0; a R software tool) was used to do dimension reduction analysis. Following that, the Python R package was used to do a visual analysis of the heat map and boxplot of the differential expression of MCODE module genes. Finally, Kaplan Meier method was used for survival analysis to obtain the most significantly different subgroups.

2.9 The Cox regression analyses of core-DEG

Based on expression profiles, using the R language Python module, a raincloud diagram (22) is utilized to graphically assess core-DEG expression differences in C3 and C4 subgroups. Then, the Cox regression analysis was used to further evaluate the relationship between the core-DEG expression and prognosis using the R software package survival and Maxstatat, and the best cutoff risk score was calculated. In addition, the Python package was used to investigate the association between various risk scores, patients' survival time, status, and gene expression changes.

2.10 Statistical analysis

For statistical analysis, R package (version 4.0.2), IBM SPSS 26.0 software and graphpad prism 8 (graphpad Software Inc, CA, USA) were utilized. All data is provided as the means ± standard deviation (SD). The Student's t test and Wilcoxon rank sum test were conducted to see if there were any differences between the sample groups. For survival analysis, the Kaplan-Meier technique was applied. Furthermore, ROC technology was adopted to assess the diagnostic effectiveness of core gene, which was represented by the Area Under Curve (AUC). The sensitivity and specificity of the gene were calculated. When the Youden's index was adjusted to its maximum value, the optimum gene cut-off value was attained. Later, the prognosis analysis was examined using Cox regression. $P<0.05$ was considered statistically significant.

3 Results

3.1 Identification of DEGs in ES

After standardizing the microarray findings, DEGs (595 in GSE45544 and 3343 in GSE17618) were discovered. According to the Venn diagram, the overlapping section of the two datasets included 187 genes (Figure 2A). There were 56 downregulated genes and 131 upregulated genes in the comparison of Ewing sarcoma tissues and noncancerous tissues (Table 1).

3.2 DEG enrichment analysis utilising GO and KEGG

DAVID was carried out to accomplish function and passage enrichment research to ascertain the biology classification of DEGs. The results were visualized using the R language pack 4.1.3 version. The DEGs were considerably enriched in the cell cycle and Staphylococcus aureus infection, according to analyzation of the KEGG pathway (Figure 2B). According to GO analysis, alterations to BPs (biological processes) in DEGs were primarily enriched in cell division, cell proliferation, cell adhesion, mitotic nuclear division, positive regulatory process of apoptosis, and drug response (Figure 2C). ATP bound, protein bound, chromatin bound, and protein kinase bound were considerably enriched in the DEGs' molecular functions (MFs) (Figure 2D). DEGs' CC (cell component) alterations were primarily enriched in the spindle pole, membrane, cytoplasm, focal adhesion, cytosol, nucleoplasm, extracellular exosome, and nucleus (Figure 2E).

3.3 Building and analyzing of the PPI network and module

Then, MCODE, a Cytoscape plugin tool, was executed to establish the most meaningful module of the DEG PPI network. The PPI network (Figure 3A) included 149 nodes and 1246 edges, with 36 genes down-regulated and 113 genes up-regulated, whereas the MCODE network (Figure 3B) was composed of 43 nodes and 857 edges. Furthermore, the biological process analysis of MCODE-DEGs was visualized by Cytoscape ClueGO (Figure 3C), which was concentrated on regulation of cyclin-dependent proteins, serine/threonine kinase activity, cytokinesis, nuclear chromosome segregation, regulation of mitotic metaphase/anaphase transition, and spindle organization. Besides, Hierarchical clustering discovered that the genes expression level of the most important module significantly distinguished the ES samples from the nontumorous samples according to the expression profiles of training sets (Figure 3D).

3.4 Core-DEG choice and evaluation

The first fifteen hub genes, which included *CCNB2*, *CCT2*, *CD44*, *ECT2*, *FOXM1*, *HLA-DPA1*, *ITGA6*, *KIF20A*, *LYZ*, *MKI67*, *PLK1*, *RFC4*, *TGFBR2*, *TYMS*, and *UBE2T*, were defined with the degree levels in the cytoHubba Cytoscape plugin, and an interaction network of the hub genes was constructed, resulting in 15 nodes and 43 edges (Figure 4A). Meanwhile, Table 2 lists the names, descriptions, and roles of these hub genes. Then, the Cytoscape ClueGO software was employed to investigate the biological processes of hub genes, which were primarily concentrated on Mitosis cytokinesis, the dTMP biological process, and the positive regulation of self-antigen tolerance induction; these data imply that hub genes have a significant function in regulating the cell cycle and homeostasis in the internal environment (Figure 4B). Furthermore, the mutated survival analyses of the hub genes was accomplished in cBioPortal online using Kaplan-Meier method. Among the 15 hub genes, only the survival analysis of *UBE2T* with and without alteration by the log-rank test demonstrated a statistically meaningful ($P < 0.05$) *UBE2T* alteration showed a significant lower overall and illness-free survival (Figure 4C), and had a poorer outcome. These data suggest that *UBE2T* may be an important biomarker in the progression of ES. As a result, *UBE2T* was defined as the "core-DEG," which will be studied later.

3.5 Expression change of UBE2T and CD99 in the validation data set and IHC of ES samples

The expression of core-DEG was validated using GSE68776. The volcanic plot displayed that 15,440 DEGs were found (up = 939,

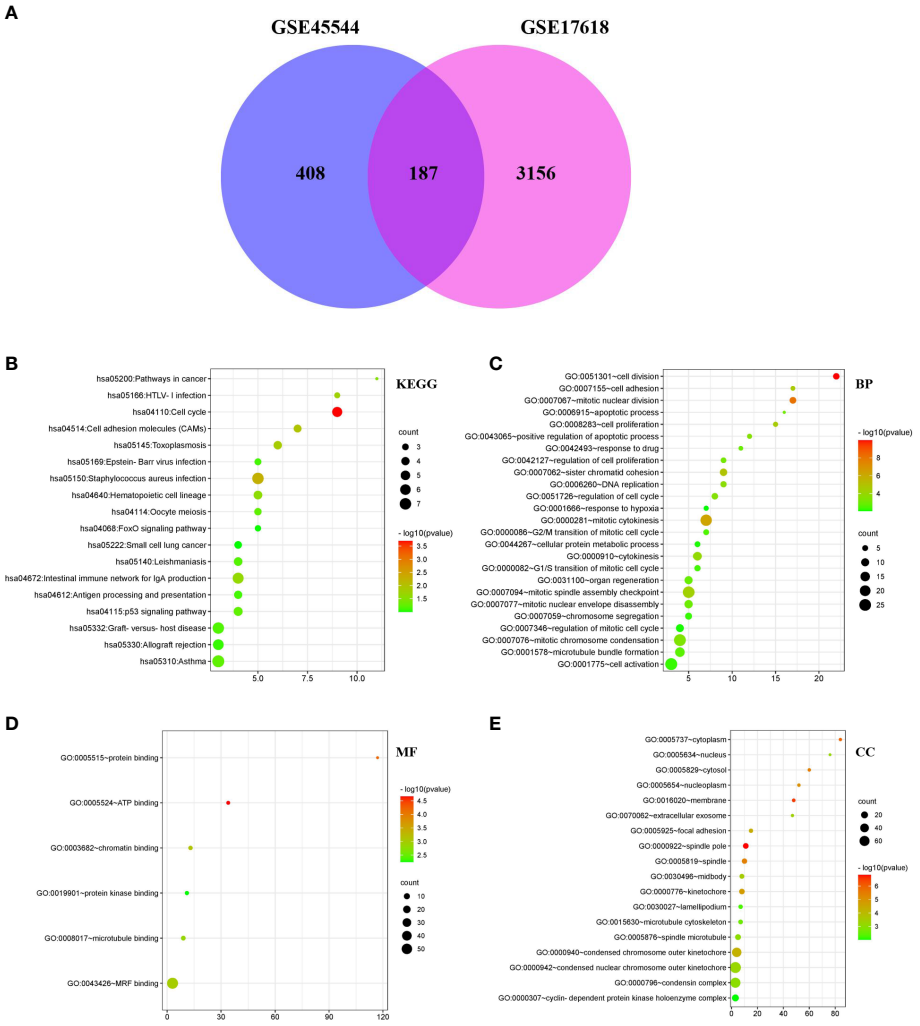


FIGURE 2
DEG Venn diagram; Bubble Plot of the GO and KEGG enrichment study for DEGs. **(A)** DEGs in the GSE17618 and GSE45544 mRNA expression profiling datasets were filtered with a fold change > 2 and a *P* value < 0.01. 187 genes overlapped between the two datasets. **(B–E)** The P value is shown by the progressively shifting hue, and the quantity of genes is denoted by the size of the black dots.

down = 6,435; |log2FC|≥1.5; adjusted *P*<0.05). *UBE2T* was significantly upregulated in validation data sets (Figure 4D). Besides, IHC was used to identify the expression of UBE2T protein in Ewing's sarcoma and normal tissues. The findings revealed that the UBE2T protein was overexpressed in ES but not in normal tissues (Figure 4E). There was a statistically significant difference between the groups (*P*<0.01) (Figure 4F). Obviously, the research data supported our prediction. Furthermore, CD99 has a high specific diagnostic value

TABLE 1 Analyzation of the datasets identified 187 DEGs, including 131 upregulated and 56 downregulated genes, in tumor samples.

Status	DEGs
Upregulated	*CD44 TPX2 CCNB1 TTC37 FRY GINS1 A2M SLCO2B1 ANLN LYZ BCLAF1 FOXM1 CHST15 PLIN2 CDC6 CST3 HOXD13 IFI16 ZNF146 RDX BDP1 SPDL1 CDK4 TYMS MELK ECT2 SNCA CDH11 NUF2 PDK4 STMN1 UBE2T CKS2 FAM84B KIF23 C1S PDLIM1 DKK3 ANKH NDRG1 MYLK CCNB2 MCM7 PRC1 CENPI JPH1 EPAS1 PMP22 KIAA0101 HLA-DPB1 BHLHE40 CELF2 NUP107 DLGAP5 MKI67 TM4SF1 PLPP3 PTPRM TOP2 EXO1 PDGFRA BHLHE41 IGK///IGKC SAT1 YPEL2 HSPA1B///HSPA1A MEF2C OAT CHPT1 VAMP8 FGL2 SQRDL ZNF704 CCT2 PAPPA SMC4 GUCY1B3 CKS1B TEAD2 GSN RHOB HLA-DPA1 EBF3 FBXO5 ZNF644 TICRR PBK PRR11 TXNIP HEATR1 ITGB3BP PRPF40A SKA3 DPT TYROBP SMC2 ASPM ATAD2 WDHD1 METTL7A BUB1B DTL TGFBR2 JAK1 LAPTM5 FAM114A1 KIF20A KCTD12 CDC5L NCAPG PLK1 RFTN1 ATP1B1 TNFSF10 CHEK1 CRYAB KIF11 SLC40A1 CD9 RFC4 TPR BUB1 BRIP1 CAD CKAP2 CXCL12 AMICAL2 FANCI CENPF NUSAP1 IGF2BP1*
Downregulated	*ABHD2 CD53 SORBS2 ALDH6A1 SUSD6 ITGA6 TPPP3 S100A16 SLC22A3 ADGRG1 MAN1A1 CECR1 RHOU SELPLG MGST2 TNFRSF21 SRPX IL10RA ENTPD1 PRELP SATB1 SYNPO2 DNAJA4 TSC22D3 RCAN2 NUPR1 TAPBP PLXNC1 TOB1 MYH11 C10orf10 LRP10 DOCK9 TLE1 MGLL CD59 PTGDS PXDC1 PEA15 SERPINB1 SERPINB6 WIPI1 RNF144B ENDOD1 ATP8A1 CA2 PPFIBP2 HLA-DMA GAS7 NEDD9 ITGA9 CTSZ CSF1R APBB1IP FRMD4B PIK3IP1*

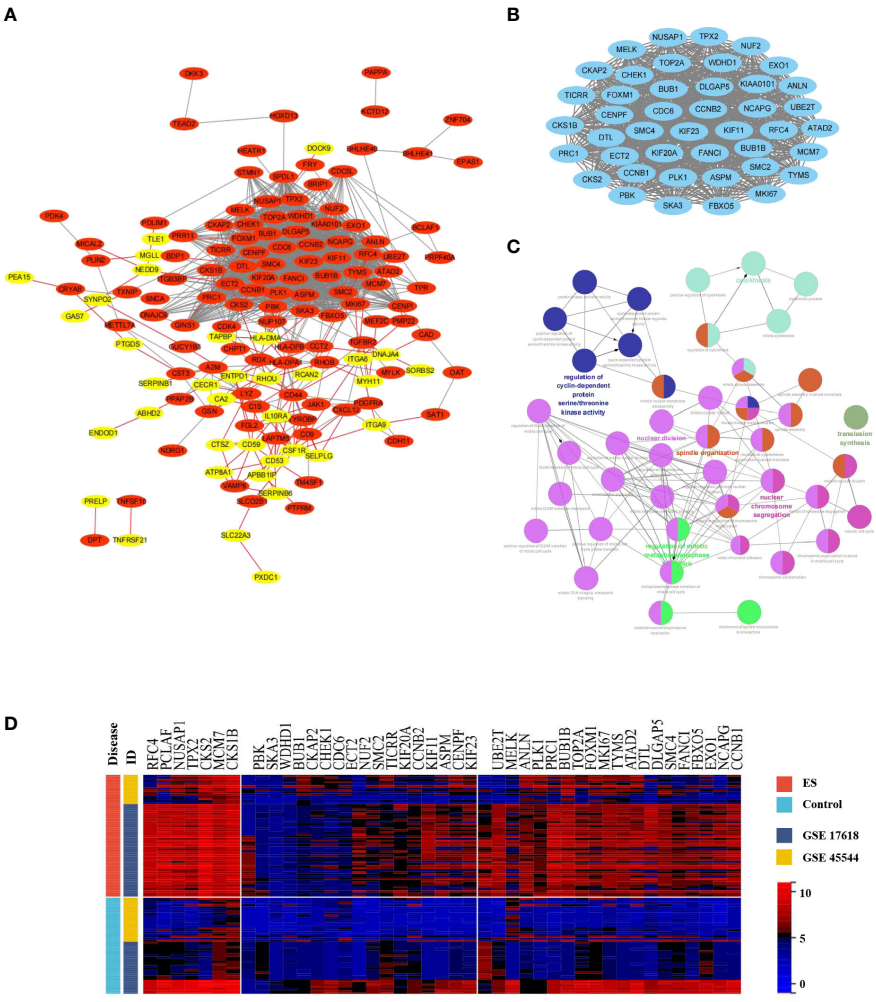


FIGURE 3
Construction of the PPI network, and MCODE module; Biology process analyzation and hierarchical clustering of MCODE-DEGs. **(A)** The Cytoscape program was built to obtain the DEGs PPI network. Genes upregulated were highlighted in light red, whereas genes downregulated were highlighted in light yellow. **(B)** The PPI network yielded the most significant module (MCODE-DEGs), having 43 nodes and 857 edges. **(C)** ClueGO was employed to evaluate the biological processes of the MCODE-DEGs $P < 0.01$ was judged statistically meaningful. The node's dark hue represented the rectified P value of ontologies. The quantity of genes participating in ontologies was represented by node's size. **(D)** The heat map demonstrated significantly different in expression levels of MCODE-DEGs between the ES and control group. Red represented upregulation of genes; Blue represented downregulation.

in IHC of ES tissue, so it is necessary to observe the difference between the ES sample and the control group. IHC results showed that CD99 was diffusely positive on the cell membrane of ES tissue (Figure 4G, H).

3.6 Diagnostic performance of *UBE2T* in the ES training set and verification set

Figure 5 depicted diagnostic value of *UBE2T* in ES. *UBE2T* was significantly overexpressed in ES in the training set (GES 17618 and GSE 45544) compared to the control group (P<0.001, Figure 5A). The Area Under Curve (AUC) of the ROC of *UBE2T* in diagnosing ES was 0.75, with sensitivity and specificity of 0.85 and 0.62, respectively (Figures 5B, C). Interestingly, the core gene is also excellent in the diagnostic evaluation of ES in the validation set (GSE68776). *UBE2T* expression was considerably increased in ES (P<0.0001, Figure 5D). The AUC of ROC was 0.90, its sensitivity was 0.94, and its specificity was 0.79 (Figures 5E, F). Obviously, these findings suggest that *UBE2T* had excellent value for ES diagnosis.

3.7 Analysis of MCODE-DEGs subgroups in ES

55 samples of ES with patients (after removing non-conformance from inclusion criteria) were divided into 4 subgroups based on the expression levels of MCODE module genes: C1(N=17), C3(N=12), C4 (N=13) and C2(N=13) (Figure 6A). Among the k = 2 to k = 10 clusters, K = 2 has the highest consistency, and k = 4 was the second (Figure 6B and Supplement figure 1). UMAP analysis indicated significant variations among the clusters (Figure 6C). Besides, The heat map revealed that the expression pattern of the MCODE-DEGs differed between the four subgroups (Figure 6D). In addition, Kaplan-Meier survival analysis showed significant differences in the

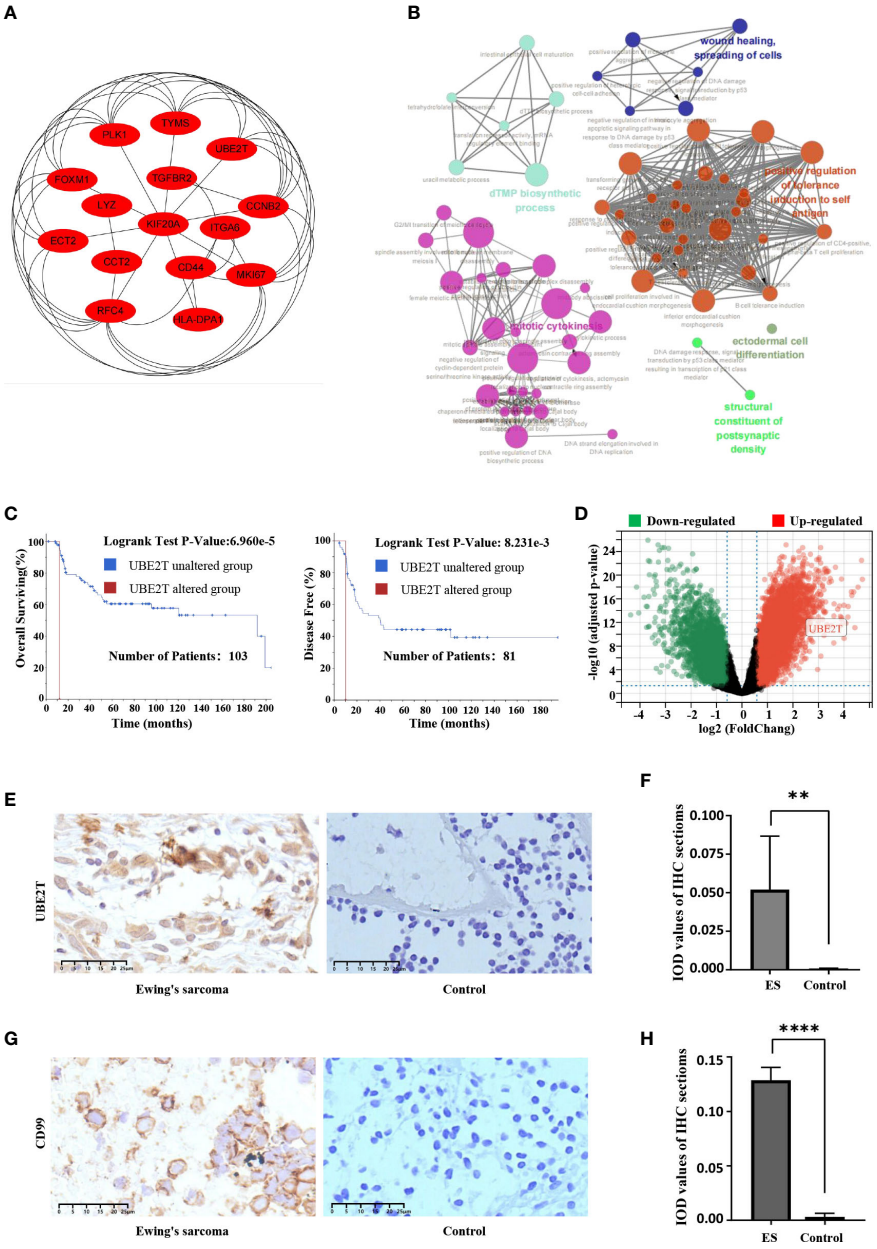


FIGURE 4
The hub genes' connection network and biological process research; Core-DEG obtained by the cBioPortal web and verified by the GSE68776 and IHC. **(A)** The hub genes were obtained by CytoHubba with 15 nodes and 43 edges. **(B)** ClueGO was utilized to examine the biological processes of hub genes. The node's dark hue represents the rectified *P* value of ontologies. The quantity of genes participating in ontologies is represented by node's size. $P < 0.01$ was judged statistically meaningful. **(C)** The cBioPortal official website was employed to complete overall surviving and illness-free surviving studies of core-DEG, $P < 0.05$. **(D)** The volcanic plot showed expression of *UBE2T* in validation set GSE68776, with |log2FC|≥1.5, adjusted $P<0.05$. **(E–H)** The IHC findings revealed that the *UBE2T* protein **(E, F)** and CD99 **(G, H)** were overexpressed in ES but not in normal tissues, and the Student's t test showed significant differences. (**$P<0.01$, ****$P<0.0001$).

subgroups (Figure 6E), especially C3 and C4, while ES patients with C3 experienced faster disease development than C4 patients. And, expression level of 34 genes in C3 (*UBE2T*, *PBK*, *CKAP2*, *CKS1B*, *WDHD1*, *CHEK1*, *CKS2*, *CCNB2*, *NCAPG*, *CENPF*, *SMC4*, *SMC2*, *BUB1*, *ECT2*, *MCM7*, *FANCI*, *ANLN*, *DTL*, *EXO1*, *CDC6*, *FBXO5*, *TYMS*, *FOXM1*, *MKI67*, *CCNB1*, *TPX2*, *ATAD2*, *PCLAF*, *NUSAP1*, *KIF23*, *TICRR*, *BUB1B*, *TOP2A* and *ASPM*) were considerably elevated compared to C4 (Figure 6F).

3.8 The correlation analysis between high expression of UBE2T and poor prognosis in ES patients

Based on training sets, a univariate Cox regression analysis was conducted to investigate the prognosis risk of core-DEGs in ES, and the results suggested that *UBE2T* was an independent risk factor ($P<0.05$, Hazard Ratio = 1.52, 95% CI) (Figure 7A). Besides, the

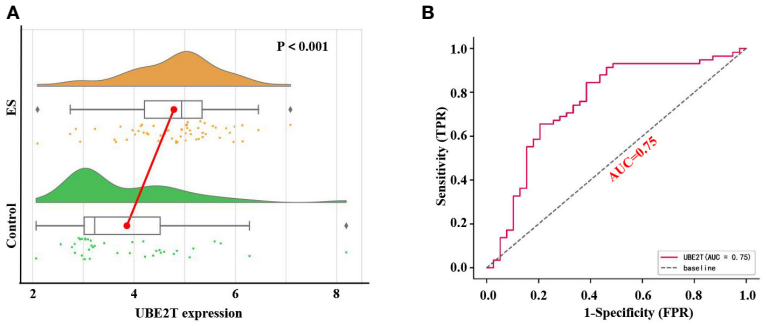
TABLE 2 Functional annotation of 15 hub genes selected by cytoHubba.

No.	Gene name	Whole name	Function
1	*CCNB2*	G2/mitotic-specific cyclin-B2	Member of the cell cycle family and is needed for cyclin regulation during the G2/M (mitosis) transition. Sub-family of cell cycle AB
2	*CCT2*	T-complex protein 1 sub-unit beta	Molecularly chaperone; aids in protein folding after ATP hydrolysis. As a component of the BBS/CCT complex, it may have a function in the formation of BBSome, a compound related to ciliogenesis that regulates vesicle transport to the cilia.
3	*CD44*	CD44 antigen	hyaluronic acid receptor (HA). Its affinity for HA, as well as its affinity for other ligands including osteopontin, collagens, and matrix metalloproteases, mediates cell-cell and cell-matrix interactions (MMPs).
4	*ECT2*	Protein ECT2	guanine nucleotide exchange factor (GEF) that catalyzes the conversion of GDP to GTP. boosts guanine nucleotide swap on Rho family small GTPase members such as RHOA, RHOC, RAC1, and CDC42.
5	*FOXM1*	Forkhead box protein M1	Transcriptional factor that regulates the expression of cyclin genes that are needed for DNA replication and mitosis.
6	*HLA-DPA1*	HLA class II histocompatibility antigen, DP alpha 1 chain	Bounds peptides produced from antigens and displays them on the cell face for identification by CD4 T-cells *via* the endocytic pathway of antigen presentation cells (APC).
7	*ITGA6*	Integrin alpha-6	Platelets have an alpha-6/beta-1 integrin receptor for laminin. Integrin alpha-6/beta-4 is a laminin receptor in epithelium cells and performs an important structural function in the hemidesmosome (By similarity).
8	*KIF20A*	Kinesin-like protein KIF20A	Mitosis kinesin is needed for cytokinesis regulated by the chromosomal passenger complex (CPC). Following PLK1 phosphorylation, implicated in PLK1 recruitment to the central spindle.
9	*LYZ*	Lysozyme C	Lysozymes are principally bacteriolysis enzymes.
10	*MKI67*	Proliferation marker protein Ki-67	After nucleal envelope destruction, this protein is needed to maintain individual mitosis chromosomes disseminated in the cytoplasm.
11	*PLK1*	Serine/threonine-protein kinase PLK1;	Serine/threonine protein kinase that regulates spindle assembly and centrosome maturity, the remove of cohesins from chromosomal arms, the deactivation of anaphase-promoting complex/cyclosome (APC/C) regulators, and the control of mitosis and cytokinesis.
12	*RFC4*	Replication Factor C subunit 4	The auxiliary proteins proliferation cell nucleal antigen (PCNA) and activator 1 are needed for the elongation of primed DNA examples by DNA polymerase delta and epsilon.
13	*TGFBR2*	TGF-beta receptor type-2	a transmembrane serine/threonine kinase that interacts with TGFBR1, the nonpromiscuous receptor for the TGF-beta cytokines TGFB1, TGFB2, and TGFB3.
14	*TYMS*	Thymidylate synthase	Adds to the route of *de novo* mitochondrion thymidylate biosynthesizing
15	*UBE2T*	Ubiquitin-conjugating enzyme E2T	It receives E1 compound ubiquitin and catalyzes its covalently binding with other proteins. Monoubiquitination is catalyzed. Mitomycin-C (MMC)-induced DNA restore. Through interaction with the E3 ubiquitin-ligase FANCL and catalytic mono-ubiquitination of FANCD2, it acts as a particular E2 ubiquitin-ligase for the Fanconi anemia complex.

Kaplan Meier survival curve demonstrated a connection between *UBE2T* expression and survival. The overall survival time of patients with high *UBE2T* expression was considerably shorter than low *UBE2T* expression (P<0.0001) (Figure 7B). In addition, the raincloud diagram showed that *UBE2T* in C3 was significantly higher than C4 (P<0.001) (Figure 7C), and the survival time was significantly shorter than that of C4 (P<0.005) (Figure 7D). These results suggest that the upregulation of *UBE2T* expression is associated with a worse outcome in ES patients. Furthermore, the study of risk score and survival time revealed that patients in the high-risk score group had considerably lower survival time than the low-risk group (P<0.0001) (Figure 7E). The findings indicated that the high-risk score group resulted in fast progression of disease. Figure 7F (including upper, middle, and lower parts) depicts the association between various risk scores, survival events, and gene expression changes. It can be shown that when *UBE2T* expression is up-regulated (Figure 7F lower part) and the risk score is increased (Figure 7F upper part), patients' survival rates decline dramatically (Figure 7F middle part). As predicted, *UBE2T* was regarded as a risk independent factor, and risk scores increased as its expression rise.

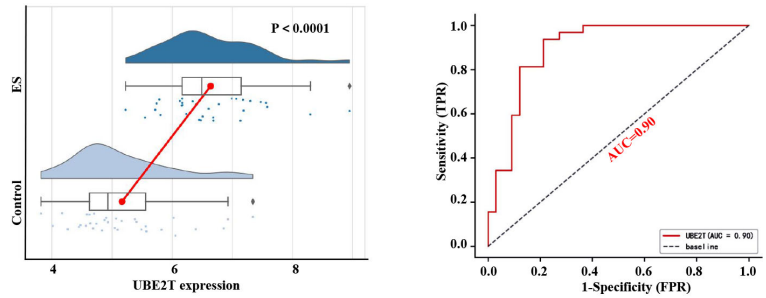
4 Discussion

Ewing sarcoma, the second commonest malignant bony neoplasm and soft-tissue malignance neoplasm in kids and teenagers, is a serious threat to human life and health (1, 2), ES is and a highly aggressive tumor with nonspecific clinical features (2). Patients with standard risk and localized disease have a 70~80% survival, and patients with metastatic disease have an approximate 30% survival (23). Previously findings have suggested that the ES family of tumors is related to immunophenotypic characteristics, chromosomal translocation (such as extraosseous ES, peripheral primitive neuroectodermal neoplasm, Askin neoplasm (24), and FET-ETS gene fusion (25, 26). Although there has



C

Marker	AUC	Sensitivity	Specificity	Youden's index	Cutoff
UBE2T	0.75	0.85	0.62	0.46	3.97



F

Marker	AUC	Sensitivity	Specificity	Youden's index	Cutoff
UBE2T	0.90	0.94	0.79	0.73	5.72

FIGURE 5
Performance of the core gene diagnostic ES in the training and verification sets. Based on the training set expression profiles (GSE17618 and GSE45544): **(A)** The difference in *UBE2T* expression between the ES and control groups. **(B)** The ROC curve of patients with ES based on the *UBE2T* gene. **(C)** The diagnostic value of the Core gene in distinguishing the ES group from the control group. According to the validation set expression profile (GSE68776): **(D)** The difference in *UBE2T* expression between the ES and control groups. **(E)** The ROC curve of people with ES based on the *UBE2T* gene. **(F)** The diagnostic value of the Core gene in distinguishing the ES group from the control group. AUC stands for Area Under the Curve; TPR stands for True Positive Rate; and FPR stands for False Positive Rate.

been improvement in the diagnosis of ES based on these preliminary studies, the specific pathogenesis remains largely unknown. Thus, it is urgent to ascertain novel biomarkers for this disease to enhance the efficiency of diagnosis and treatment. Microarray technology is beneficial for investigating genetic abnormalities for ES, which may be of benefit for the corroboration of novelty biomarkers to contribute to the improvement of early diagnosing and prediction prognosis for ES.

In the current investigation, two microarray datasets were selected from GEO, and bioinformatics analyzation was run to discover DEGs between ES tissues and nontumorous tissues. In all 187 DEGs were identified through analysis and comparison of those two datasets, including 56 downregulation genes and 131 upregulation. GO and KEGG enrichment analyzation were used to investigate interrelations in the DEGs. The up-regulation genes were majorly concentrated in cell dividing, mitosis nucleus dividing, proliferation, apoptotic process, response to drug, and positive regulation of apoptotic process, whereas this downregulation genes were primarily enriched in cell adhesion (Table 3). Life involves constant changes, and the cell cycle is required to maintain cell growth and DNA duplication, followed by cell division (mitosis), proliferation, and apoptosis. Remarkably, the cell cycle has an important effect on maintaining the normal process of life. Thus, dysregulation of the cell cycle process is closely related to the carcinogenesis or progression of tumors (27–29). In addition, recent reports have shown that the molecular mechanism of cell adhesion has a significant effect on collective cancer cell migrating, and mutations and changes in cell adhesion protein expression are frequently related to tumorous progression (30, 31). Whats more, changes in the tumor microenvironment may affect immune cell regulation (32). Our research findings revealed that, according to the Cytoscape ClueGO analysis, the biological processes of hub genes gathered in Mitosis

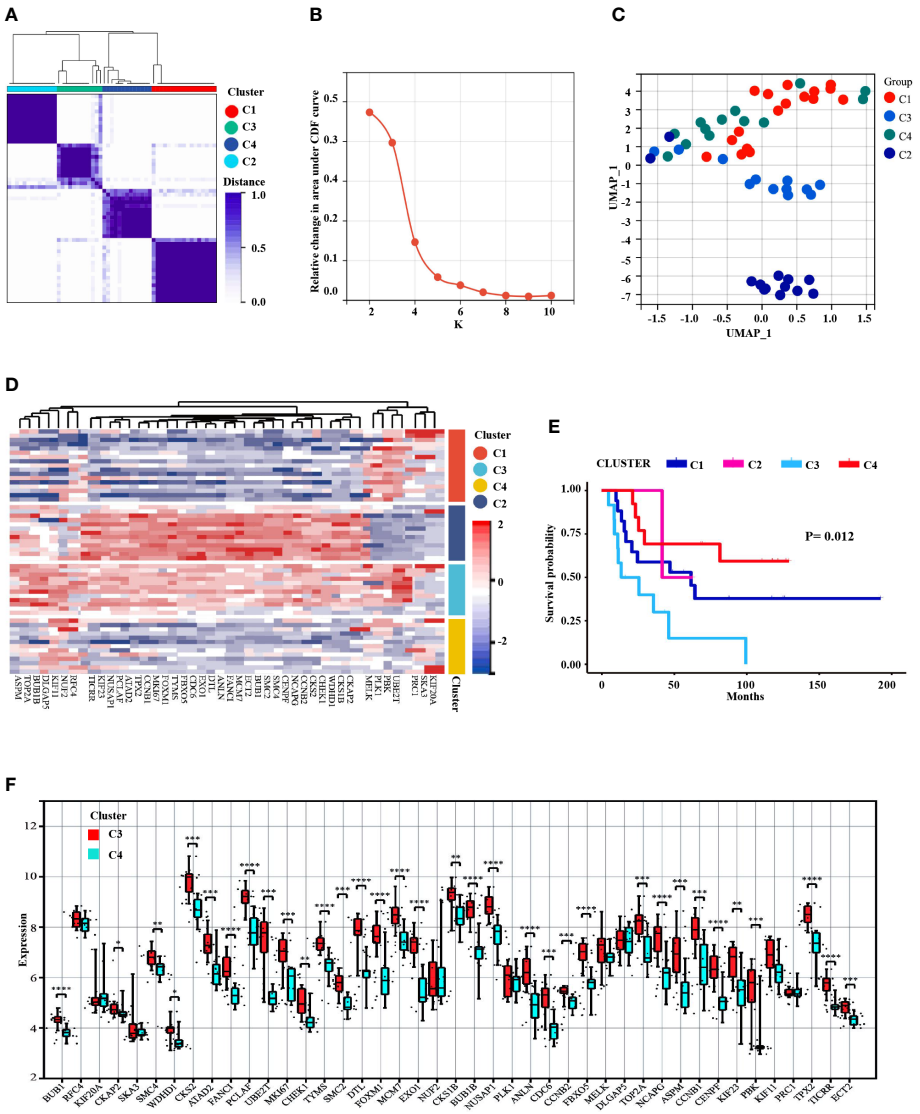


FIGURE 6
Prognosis identification of ES patients by clustering analysis based on MCODE-DEGs expression profifile. **(A)** Consensus clustering divided into 4 subgroups. **(B)** The CDF curve showed the consistency of clustering (K=2 the highest consistency, followed by K4). **(C)** UMAP dimension reduction analysis testified the classification. **(D)** The heat map displayed the different expression patterns of the MCODE-DEGs in 4 clusters. **(E)** The survival analysis of ES patients found significant differences among the four subgroups, $P<0.05$. **(F)** Boxplot revealed difference expression status of the MCODE-DEGs between C3 and C4 (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

cytokinesis, the dTMP biological process, and the positive regulation of self-antigen tolerance induction, which maintained stability of the cell cycle and the internal environment.

Beside, In total, 15 hub genes were extracted relied on the most significant module with the degree rank (Figure 4A). One of these hub genes, ubiquitin-conjugating enzyme E2 T (*UBE2T*), catalyzes monoubiquitination, which is a significant posttranslational modification that affects a variety of biological activities, for instance, immune reactions, inflammation, cell proliferation, and cell differentiation (33–36). Interestingly, *UBE2T* plays an essential part in the DNA damage pathway, and it has been demonstrated to be correlated intimately with the development and poor prognosis of several cancers, such as gastric cancer, hepatocellular cancer, prostate cancer, and gallbladder cancer (37–40). Upregulation of *UBE2T* levels has been disclosed to enhance gastric cancer development through *RACK1* ubiquitination, and a novel powerful *UBE2T* inhibitor has been identified to suppress gastric cancer progression by blocking RACK1 ubiquitination after aberrant Wnt/β-catenin signaling (40). Moreover, Sun et al. (38) discovered that *UBE2T* was increased in HCC tissues, and that HCC sufferers with greater *UBE2T* quantities have a worse prognosis, demonstrating that *UBE2T*-regulated *H2AX* mono-ubiquitination may induce hepatocellular carcinoma radiation resistance by boosting *CHK1* activation. In addition, previous studies showed that the vulnerability of anticancer drugs is based on the involvement of proteins in ubiquitination and degradation, which provides a theoretical basis for the development of therapeutic drugs with genome modification (41, 42). As a result, *UBE2T* may be regarded as a therapeutic potential target for ES sufferers' therapy.

However, there are few reports on the relationship between *UBE2T* and ES. Therefore, the present study analyzed several ES

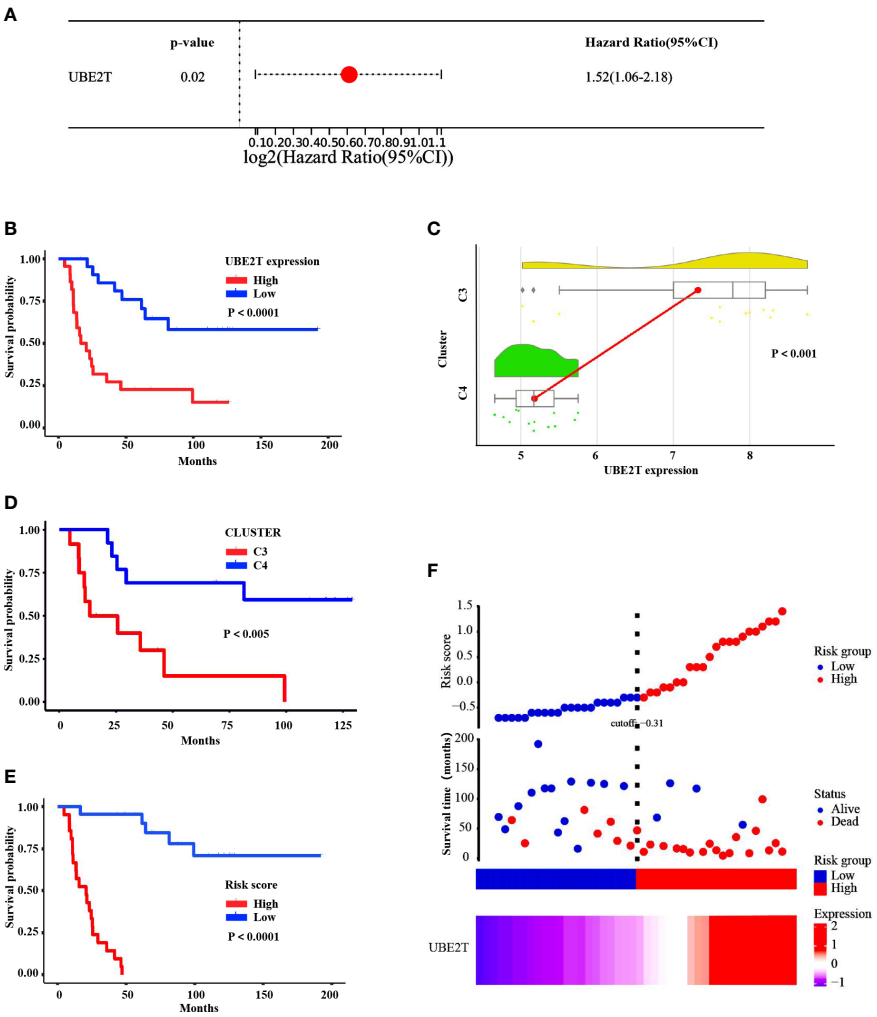


FIGURE 7
Correlation analysis between expression of *UBE2T* and prognosis in ES patients of the training cohorts. **(A)** HR and 95% CI of the core DEGs based on a unitvariable Cox regression analysis. **(B)** The Kaplan Meier survival curve demonstrated that the overall survival time of patients with *UBE2T* high expression was evidently shorter than low expression (*P*<0.0001). **(C)** The raincloud diagram showed the expression of *UBE2T* in C3 was significantly higher than C4 (*P*<0.001). **(D)** The K-M survival curve indicated that the survival time of C3 was significantly shorter than C4 (*P*<0.005). **(E)** The K-M survival curve displayed that patients in the high-risk score group had considerably lower survival time than the low-risk group (*P*<0.0001). **(F)** The distribution of risk score, survival status, and *UBE2T* expression level revealed that risk score increased as *UBE2T* expression increased, while survival rate decreased dramatically.

datasets in the GEO data bank and discovered that *UBE2T* expression was observed to be considerably greater in tumor samples than in nontumor samples. Furthermore, validation set and IHC findings displayed that the expression level of UBE2T was significantly higher in the sick tissues of Ewing's sarcoma patients than the control group, and IHC analysis revealed that UBE2T was mostly expressed in the cytoplasm of Ewing's sarcoma cells (Figures 4E, F). These results are consistent with our predictions. In addition, the investigation on the diagnostic value of core genes in ES observed that the AUC of *UBE2T* had excellent performance in both the training group and the verification group (Figure 5). Following that, we explored the relationship between the expression level of the *UBE2T* and prognosis by Cox regression and K-M survival analasis in ES patients according to the expression profiles of training sets. The findings revealed that *UBE2T* was an independent risk factor (Figure 7A), and patients with high expression of the *UBE2T* and the high-risk score, which led to a poor prognosis, had a negatively correlated survival time (Figures 7B–E). As a result, based on the above findings, this study demonstrated that *UBE2T* can be seen as an important value biomarker for diagnosis and treatment of ES, thereby providing a new potential therapeutic target for ES as well as an important new perspective for evaluating the effect of treatment and prognostic prediction.

5 Conclusion

In summary, the current examination found that *UBE2T* expression was greater in tumor tissues from ES patients than in non-tumor tissues and that *UBE2T* had an important value as a biomarker for the diagnosis of ES. Furthermore, increased *UBE2T* expression is associated with a terrible prognosis. As a result, *UBE2T* can be exploited as an independent prognostic biomarker in patients with ES. However, the existing research has drawbacks. First, consider

TABLE 3 Enrichment investigation of positive-regulation and negative-gulation genes with DEGs in ES specimens employing GO and KEGG Pathway.

Term	Description	Count	P value
Upregulation			
GO:0051301	cell division	21	8.53E-13
GO:0007067	mitotic nuclear division	16	3.43E-10
GO:0008283	cell proliferation	14	2.61E-06
GO:0006915	apoptotic process	13	8.67E-04
GO:0042493	response to drug	11	7.52E-05
GO:0043065	positive regulation of apoptotic process	10	3.44E-04
Hsa04110	cell cycle	9	1.50E-05
Hsa05166	HTLV-I infection	8	0.008161847
Downregulation			
GO:0007155	cell adhesion	9	7.67E-05
Hsa04514	cell adhesion molecules (CAMs)	4	0.015820009

the patient sample size constraints. As a consequence, *UBE2T* research should be added to the wider ES queue. Second, this study only investigated at *UBE2T* expression level in tumor tissues and did not researched *UBE2T* functionality *in vivo* or *in vitro*. As a result, further tests and investigations are required to uncover the potential mechanism of *UBE2T* in ES.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

GQ: Collection, organizing, and review of the literature, preparing the manuscript, manuscript review and modification. YX and YQ: Samples collection, immunohistochemistry, bioinformatics analysis and revision of the manuscript. JQ and GC: Data processing and pictures arrangement. NZ and JD: Data analysis, editing of manuscript and revision. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

This study was supported by the projects of the State Key Laboratory of Trauma, Burns and Combined Injury of China (SKLKF201802, SKLYQ201901), the National Natural Science Foundation of China (81971830 and 82260372), applied research on the construction and clinical promotion of the trauma grading treatment system in Guizhou Province in China (Qian Science Cooperation SY word [2015] No. 3041), Science and Technology Innovation Talent Team of Critical Care and Green Channel of Guizhou Province (No: [2017] 5654), and the Health Industry Research Project of Hainan Province in China (20A200347). All authors thank the contributors of the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and TCGA database (https://tcga-data.nci.nih.gov/) for sharing their data on open access, and the Sangerbox (http://sangerbox.com) and Micro-bioinformatic (http://www.bioinformatics.com) for offering visualization analysis.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1000949/full#supplementary-material

References

1. Balamuth NJ, Womer RB. Ewing's sarcoma. *Lancet Oncol* (2010) 11:184–92. doi: 10.1016/S1470-2045(09)70286-4

2. Riggi N, Suvà ML, Stamenkovic I. Ewing's sarcoma. *New Engl J Med* (2021) 384:154–64. doi: 10.1056/NEJMra2028910

3. Pappo AS, Dirksen U. Rhabdomyosarcoma, Ewing sarcoma, and other round cell sarcomas. *J Clin Oncol* (2018) 36:168–79. doi: 10.1200/JCO.2017.74.7402

4. Brasme JF, Morfouace M, Grill J, Martinot A, Amalberti R, Bons-Letouzey C, et al. Delays in diagnosis of paediatric cancers: A systematic review and comparison with expert testimony in lawsuits. *Lancet Oncol* (2012) 13:e445–459. doi: 10.1016/S1470-2045(12)70361-3

5. Gaspar N, Hawkins DS, Dirksen U, Lewis IJ, Ferrari S, Le Deley MC, et al. Ewing Sarcoma: Current management and future approaches through collaboration. *J Clin Oncol* (2015) 33:3036–46. doi: 10.1200/JCO.2014.59.5256

6. Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* (2002) 30:207–10. doi: 10.1093/nar/30.1.207

7. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: Tool for the unification of biology. the gene ontology consortium. *Nat Genet* (2000) 25:25–9. doi: 10.1038/75556

8. Consortium TGO. The gene ontology resource: Enriching a GOld mine. *Nucleic Acids Res* (2021) 49:D325–34. doi: 10.1093/nar/gkaa1113

9. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* (2000) 28:27–30. doi: 10.1093/nar/28.1.27

10. Savola S, Klami A, Myllykangas S, Manara C, Scotlandi K, Picci P, et al. High expression of complement component 5 (C5) at tumor site associates with superior survival in ewing's sarcoma family of tumour patients. *ISRN Oncol* (2011) 2011:168712. doi: 10.5402/2011/168712

11. Specht K, Sung YS, Zhang L, Richter GH, Fletcher CD, Antonescu CR. Distinct transcriptional signature and immunoprofile of CIC-DUX4 fusion-positive round cell tumors compared to EWSR1-rearranged Ewing sarcomas: Further evidence toward distinct pathologic entities. *Genes Chromosomes Cancer* (2014) 53:622–33. doi: 10.1002/gcc.22172

12. Svoboda LK, Harris A, Bailey NJ, Schwentner R, Tomazou E, von Levetzow C, et al. Overexpression of HOX genes is prevalent in Ewing sarcoma and is associated with altered epigenetic regulation of developmental transcription programs. *Epigenetics* (2014) 9:1613–25. doi: 10.4161/15592294.2014.988048

13. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* (2009) 37:1–13. doi: 10.1093/nar/gkn923

14. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* (2017) 45:D353–61. doi: 10.1093/nar/gkw1092

15. Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, et al. The STRING database in 2021: Customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res* (2021) 49:D605–12. doi: 10.1093/nar/gkaa1074

16. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* (2003) 13:2498–504. doi: 10.1101/gr.1239303

17. Bandettini WP, Kellman P, Mancini C, Booker OJ, Vasu S, Leung SW, et al. MultiContrast delayed enhancement (MCODE) improves detection of subendocardial myocardial infarction by late gadolinium enhancement cardiovascular magnetic resonance: A clinical validation study. *J Cardiovasc Magnetic Resonance* (2012) 14:83. doi: 10.1186/1532-429X-14-83

18. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: A cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* (2009) 25:1091–3. doi: 10.1093/bioinformatics/btp101

19. Chin CH, Chen SH, Wu HH, Ho CW, Ko MT, Lin CY. cytoHubba: Identifying hub objects and sub-networks from complex interactome. *BMC Syst Biol* (2014) 8 Suppl 4:S11. doi: 10.1186/1752-0509-8-S4-S11

20. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discovery* (2012) 2:401–4. doi: 10.1158/2159-8290.CD-12-0095

21. Wilkerson MD, Hayes DN. ConsensusClusterPlus: A class discovery tool with confidence assessments and item tracking. *Bioinformatics* (2010) 26:1572–3. doi: 10.1093/bioinformatics/btq170

22. Allen M, Poggiali D, Whitaker K, Marshall TR, Kievit RA. Raincloud plots: A multi-platform tool for robust data visualization. *Wellcome Open Res* (2019) 4:63. doi: 10.12688/wellcomeopenres.15191.1

23. Grünewald T, Cidre-Aranaz F, Surdez D, Tomazou EM, de Álava E, Kovar H, et al. Ewing Sarcoma. *Nat Rev Dis Primers* (2018) 4:5. doi: 10.1038/s41572-018-0003-x

24. Kovar H. Ewing's sarcoma and peripheral primitive neuroectodermal tumors after their genetic union. *Curr Opin Oncol* (1998) 10:334–42. doi: 10.1097/00001622-199807000-00010

25. Watson S, Perrin V, Guillemot D, Reynaud S, Coindre JM, Karanian M, et al. Transcriptomic definition of molecular subgroups of small round cell sarcomas. *J Pathol* (2018) 245:29–40. doi: 10.1002/path.5053

26. Parcesepe P, Giordano G, Zanella C, Giuliani J, Greco F, Bonetti A, et al. Colonic Ewing Sarcoma/PNET associated with liver metastases: A systematic review and case report. *Pathol Res Pract* (2019) 215:387–91. doi: 10.1016/j.prp.2018.11.021

27. Tripathi V, Shen Z, Chakraborty A, Giri S, Freier SM, Wu X, et al. Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor b-MYB. *PloS Genet* (2013) 9:e1003368. doi: 10.1371/journal.pgen.1003368

28. Shostak A. Circadian clock, cell division, and cancer: From molecules to organism. *Int J Mol Sci* (2017) 18(4):873. doi: 10.3390/ijms18040873

29. Venuto S, Merla G. E3 ubiquitin ligase TRIM proteins, cell cycle and mitosis. *Cells* (2019) 8(5):510. doi: 10.3390/cells8050510

30. Läubli H, Borsig L. Altered cell adhesion and glycosylation promote cancer immune suppression and metastasis. *Front Immunol* (2019) 10(2120). doi: 10.3389/fimmu.2019.02120

31. Janiszewska M, Primi MC, Izard T. Cell adhesion in cancer: Beyond the migration of single cells. *J Biol Chem* (2020) 295:2495–505. doi: 10.1074/jbc.REV119.007759

32. Yang L, Liu Y, Zhang BK, Yu MS, Huang F, Wen Y, et al. Prognostic tumor microenvironment gene and the relationship with immune infiltration characteristics in metastatic breast cancer. *Biocell* (2022) 46(5):1215–43. doi: 10.32604/biocell.2022.018221

33. Alpi AF, Chaugule V, Walden H. Mechanism and disease association of E2-conjugating enzymes: Lessons from UBE2T and UBE2L3. *Biochem J* (2016) 473:3401–19. doi: 10.1042/BCJ20160028

34. Qiu J, Sheedlo MJ, Yu K, Tan Y, Nakayasu ES, Das C, et al. Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. *Nature* (2016) 533:120–4. doi: 10.1038/nature17657

35. Kao SH, Wu HT, Wu KJ. Ubiquitination by HUWE1 in tumorigenesis and beyond. *J Biomed Sci* (2018) 25:67. doi: 10.1186/s12929-018-0470-0

36. Yang L, Guo W, Zhang S, Wang G. Ubiquitination-proteasome system: A new player in the pathogenesis of psoriasis and clinical implications. *J Dermatol Sci* (2018) 89:219–25. doi: 10.1016/j.jdermsci.2017.12.002

37. Wen M, Kwon Y, Wang Y, Mao JH, Wei G. Elevated expression of UBE2T exhibits oncogenic properties in human prostate cancer. *Oncotarget* (2015) 6:25226–39. doi: 10.18632/oncotarget.4712

38. Sun J, Zhu Z, Li W, Shen M, Cao C, Sun Q, et al. UBE2T-regulated H2AX monoubiquitination induces hepatocellular carcinoma radioresistance by facilitating CHK1 activation. *J Exp Clin Cancer Res* (2020) 39:222. doi: 10.1186/s13046-020-01734-4

39. Zhu X, Li T, Niu X, Chen L, Ge C. Identification of UBE2T as an independent prognostic biomarker for gallbladder cancer. *Oncol Lett* (2020) 20:44. doi: 10.3892/ol.2020.12230

40. Yu Z, Jiang X, Qin L, Deng H, Wang J, Ren W, et al. A novel UBE2T inhibitor suppresses wnt/β-catenin signaling hyperactivation and gastric cancer progression by blocking RACK1 ubiquitination. *Oncogene* (2021) 40:1027–42. doi: 10.1038/s41388-020-01572-w

41. Garraway LA, Verweij J, Ballman KV. Precision oncology: An overview. *J Clin Oncol* (2013) 31:1803–5. doi: 10.1200/JCO.2013.49.4799

42. Pagliarini R, Shao W, Sellers WR. Oncogene addiction: Pathways of therapeutic response, resistance, and road maps toward a cure. *EMBO Rep* (2015) 16:280–96. doi: 10.15252/embr.201439949

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 21 February 2023
DOI 10.3389/fonc.2023.1064190

OPEN ACCESS

EDITED BY
Jilong Yang,
Tianjin Medical University Cancer Institute and Hospital, China

REVIEWED BY
Ho Joon Im,
Asan Medical Center, Republic of Korea
Meng Lv,
Peking University People's Hospital, China
Suparno Chakrabarti,
Narayana Health, India

*CORRESPONDENCE
Thomas Eichholz
thomas.eichholz@med.uni-tuebingen.de

SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 07 October 2022
ACCEPTED 01 February 2023
PUBLISHED 21 February 2023

CITATION
Eichholz T, Döring M, Giardino S, Gruhn B, Seitz C, Flaadt T, Schwinger W, Ebinger M, Holzer U, Mezger M, Teltschik H-M, Sparber-Sauer M, Koscielniak E, Abele M, Handgretinger R and Lang P (2023) Haploidentical hematopoietic stem cell transplantation as individual treatment option in pediatric patients with very high-risk sarcomas. *Front. Oncol.* 13:1064190. doi: 10.3389/fonc.2023.1064190

Haploidentical hematopoietic stem cell transplantation as individual treatment option in pediatric patients with very high-risk sarcomas

Thomas Eichholz[1]*, Michaela Döring[1], Stefano Giardino[2], Bernd Gruhn[3], Christian Seitz[1], Tim Flaadt[1], Wolfgang Schwinger[4], Martin Ebinger[1], Ursula Holzer[1], Markus Mezger[1], Heiko-Manuel Teltschik[5], Monika Sparber-Sauer[5,6], Ewa Koscielniak[5,6], Michael Abele[1], Rupert Handgretinger[1] and Peter Lang[1]

[1]University Children's Hospital, Eberhard Karls University, Tuebingen, Germany, [2]Hematopoietic Stem Cell Transplantation Unit, Department of Hematology and Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [3]Department of Pediatrics, Jena University Hospital, Jena, Germany, [4]Department of Pediatrics and Adolescent Medicine, Medical University of Graz, Graz, Austria, [5]Klinikum der Landeshauptstadt Stuttgart gKAöR, Olgahospital, Stuttgart Cancer Center, Zentrum für Kinder-, Jugend- und Frauenmedizin, Pädiatrie 5 (Pädiatrische Onkologie, Hämatologie, Immunologie), Stuttgart, Germany, [6]University Tübingen, Medical Faculty, Tübingen, Germany

Background: Prognosis of children with primary disseminated or metastatic relapsed sarcomas remains dismal despite intensification of conventional therapies including high-dose chemotherapy. Since haploidentical hematopoietic stem cell transplantation (haplo-HSCT) is effective in the treatment of hematological malignancies by mediating a graft versus leukemia effect, we evaluated this approach in pediatric sarcomas as well.

Methods: Patients with bone Ewing sarcoma or soft tissue sarcoma who received haplo-HSCT as part of clinical trials using CD3+ or TCRα/β+ and CD19+ depletion respectively were evaluated regarding feasibility of treatment and survival.

Results: We identified 15 patients with primary disseminated disease and 14 with metastatic relapse who were transplanted from a haploidentical donor to improve prognosis. Three-year event-free survival (EFS) was 18,1% and predominantly determined by disease relapse. Survival depended on response to pre-transplant therapy (3y-EFS of patients in complete or very good partial response: 36,4%). However, no patient with metastatic relapse could be rescued.

Conclusion: Haplo-HSCT for consolidation after conventional therapy seems to be of interest for some, but not for the majority of patients with high-risk pediatric sarcomas. Evaluation of its future use as basis for subsequent humoral or cellular immunotherapies is necessary.

KEYWORDS

haploidentical hematopoietic stem cell transplantation, pediatric sarcoma, Ewing sarcoma, soft tissue sarcoma, rhabdomyosarcoma

Introduction

Ewing sarcoma (ES) together with soft tissue and other extraosseous sarcomas accounted for 8% of malignancies in children <18 years between 2009 and 2018 in Germany (1). Despite advanced multimodal therapies, the prognosis of these entities remains poor in the case of primary disseminated or relapsed disease.

The five-years overall survival (OS) of children with disseminated rhabdomyosarcoma (RMS) is estimated between 20-30% (2, 3), reducing to less than 20% in relapsed disease (4). Nevertheless, this group of patients is not uniform and in presence of recognized risk factors such as the age older than 10 years with bone or bone marrow metastasis, the 5-year EFS falls lower than 2% (5). Alveolar histology, age of over 10 or below 1 year, bone or bone marrow metastasis, and the site of primary tumor have been also found to affect prognosis (2, 6). These conditions enter into the Oberlin-score, which defines different prognostic subgroups. An Oberlin score >2 is associated with a 3-year EFS of 14% (7).

Similarly, the prognosis of patients with disseminated or early relapsed ES is poor, with a 5-year OS reported lower than 15% (8). The age of over 14 years, bone metastasis and multiple metastases, initial tumor volume >200ml (9) and relapse within two years (10) in ES are the recognized unfavorable prognostic parameters.

For other non-rhabdomyosarcoma soft tissue sarcomas (NRSTS), like synovial sarcoma, unsatisfactory survival rates have been reported in Stage IV (11) or relapsed disease (12) as well, making them eligible for experimental therapies (13).

Despite their chemosensitivity, dose escalation with autologous stem cell rescue did not clearly result in better survival in RMS or ES. While one study found a better survival of high-dose chemotherapy in high-risk localized ES (14), other studies did not verify an improvement in survival either in RMS (6), nor ES (15, 16), and new alternative treatment options are needed in very high-risk situations for relapsed disease with very poor prognosis.

Since a graft-versus-tumor (GvT) effect could be shown in some other solid tumors (17), also in pediatric sarcomas allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been seen as a potential alternative treatment option (18). Different case reports have suggested a GvT effect even in pediatric RMS and ES (5, 19–23), supporting this strategy. The development of efficient graft manipulation techniques and a reduced intensity conditioning regimen (RIC) lead to overcoming the main risks for transplant-related mortality even in haploidentical settings (24, 25), by potentially facilitating a higher GvT effect (24, 26) mediated by cell subsets preserved in the T-depleted grafts and avoiding the limiting post-transplant immunosuppressive treatments. An antitumoral effect of natural killer (NK) – cells against pediatric solid tumor cells in haploidentical setting was shown *in vitro* (27).

Here we present a retrospective analysis of children affected by RMS, ES or NRSTS who underwent an HSCT from a haploidentical donor after T-cell negative selection in 3 centers (Tübingen, Jena, and Graz).

Materials and methods

Data from children with a diagnosis of RMS, ES or NRSTS who underwent haplo-HSCT from 2005 to 2019 have been retrospectively collected. Patients were enrolled in clinical trials about haplo-HSCT with T-cell depletion in pediatric diseases in that period with CD3+/CD19+ (Tübingen, Jena, Graz) [ClinicalTrials.gov NCT01919866] or TCRα/β+ and CD19+ negative selection (Tübingen). Patients with RMS, ES or NRSTS and primary bone metastasis or bone marrow involvement, with relapsed metastatic or primary refractory disease to standard treatment were eligible for haplo-HSCT. Patients have been in part already published in another context (28, 29). Data analysis was done as of August 2022.

The patients have been considered eligible for this analysis in presence of a diagnosis ES, RMS or other STS at high risk because of stage IV disease at diagnosis or relapse. Data regarding diagnosis, disease status before transplant, pre-HSCT treatments, conditioning regimen, donor features, graft manipulation, engraftment, transplant-related toxicity/morbidity, acute (aGvHD) and chronic graft-versus-host disease (cGvHD), disease status after HSCT, and survival have been retrospectively collected if not already present in the databases of the above-mentioned clinical trials.

We evaluated overall survival (OS) and event-free survival (EFS) as well as the incidence of transplant-related mortality (TRM), grade II-IV aGvHD, extensive cGvHD and relapse.

Data analysis and definitions: Stage IV disease has been defined as the presence of distant metastasis at diagnosis. Complete response was defined as the disappearance of all visible disease on imaging, partial response (PR) as a reduction of at least 30% (ES) or 33% (RMS/NRSTS) of tumor volume and progressive disease (PD) as an increase in tumor volume of more than 20% (ES) or 33% (RMS/NRSTS) or the appearance of new lesions according to the respective treatment protocols. Nonresponse was defined as a tumor response between PR and PD. Very good partial response (VGPR) was defined as a reduction of ≥90 of tumor volume or the persistence of unclear residuals upon imaging. Engraftment was defined as the first of three consecutive days with an absolute leukocyte count (ALC) of more than 1000/µl. GvHD was diagnosed and graded according to Glucksberg criteria (30). A failure to achieve ALC >1000/µl until day 28 posttransplant defined primary graft failure (PGF), whereas a decline of ALC <1000/µl after initial engraftment and not caused by infection, drug toxicity or relapse defined secondary graft failure.

The probability of survival from the date of transplantation to death/last follow-up was defined as overall survival (OS). In one patient who was rescued with a syngeneic stem cell graft after rejection of her haploidentical graft, TRM, cumulative incidence of relapse, GvHD or virus reactivation, EFS and OS were censored at the timepoint of subsequent transplantation. Every death, not caused by relapse or progress of the underlying disease was accounted as transplant-related. Events were defined as relapse, death in remission or secondary malignancy, whichever occurred first. The Kaplan-Meier method was used to estimate the probability of survival, using GraphPad Prism version 7 for Windows software (GraphPad Software, Inc., San Diego, California USA, www.graphpad.com). Calculation of cumulative incidence of time to relapse, time to ADV or CMV virus reactivation, time to GvHD and time to treatment related death accounting for the concurrent risk "death", "relapse" and "non-treatment related death", respectively were done by SAS using Cox regression.

Results

We identified 29 patients eligible for this analysis, with diagnoses of ES (n=14), alveolar rhabdomyosarcoma (aRMS) (n=12), botryoid

rhabdomyosarcoma (n=1), undifferentiated sarcoma (n=1) or synovial sarcoma (n=1) who received haplo-HSCT in centers involved (Tübingen n=23, Jena n=5, Graz n=1).

All patients were considered to be at very high risk due to stage IV disease at diagnosis (n=23) and/or relapse (n=14) and eligible for haplo-HSCT. In all but one patient with aRMS, the Oberlin-score was ≥2. Eight patients with ES presented with multiple bone metastasis at diagnosis and 8 relapsed within 2 years after initial diagnosis, before they were considered for haplo-HSCT. Patient characteristics are summarized in Table 1.

Initial therapy complied with the ongoing clinical trials and recommendations at the time of diagnosis. Patients with ES were treated either according Euro-E.W.I.N.G.99 or EWING-2008 study respectively. All but two patients with RMS, as well as the patients with unspecified sarcoma and synovial sarcoma were treated according to the studies and recommendations of the CWS study group, including CWS-96, CWS IV-2002, CWS DOK IV 2004, CWS-2002P and CWS guidance. Of the remaining two patients with rhabdomyosarcoma, one received initial treatment in the United States according to the recommendations of the children´s oncology group (COG). The other one was treated according to EWING-2008, since the primary diagnosis was ES but changed to aRMS after relapse. The majority of patients (15; 51,7%) responded to initial therapy and entered transplantation in first complete remission (CR1, n=6), very good partial response (VGPR1, n=3) or partial response (PR1, n=6). Another 8 patients (27,6%) responded after previous relapse or progression (CR≥2 n=2; PR≥2 n=6). Six patients were transplanted without response (NR) (20,7%).

Conditioning regimen: Apart from treosulfan- and busulfan-based conditioning regimens in two and one patient, a toxicity reduced regimen was deployed in most transplants, using fludarabin (160 mg/m² body surface area) in 24 or clofarabin (200mg/m²/d) in 2 cases, in

TABLE 1 Patient Characteristics.

	Total	Ewing Sarcoma	Rhabdomyosarcoma	Non-rhabdomyosarcoma soft tissue sarcoma
No of patients (%)	29	14 (48,3%)	Alveolar RMS 12 (41,4%) Botryoid RMS 1 (3,4%)	Undifferentiated sarcoma 1 (3,4%) Synovial sarcoma 1 (3,4%)
Age at diagnosis	12,2	12,9	7,5	10,96
years median (range)	(1 – 23,8)	(7,9 – 17,7)	(1 – 23,8)	(10,2 – 11,8)
Diagnosis (%)				
• Primary stage IV	15 (51,7%)	5	9	1
• Metastatic relapse	14 (48,3%)	9	4	1
Previous therapies				
• Radiotherapy	19 (65,5%)	10	7	2
• Autologous/syngeneic HSCT	9 (31%)	7	2	0
• other	Samarium 3	Samarium 1	Samarium 2	
	mTOR Inhibitor 3	mTOR Inhibitor 2	mTOR Inhibitor 1	
	TKI 1	TKI 1		
	Anti-IGF2-mAb 1	Anti-IGF2-mAb 1		
	Avastin 1		Avastin 1	
	HIPEC 1		HIPEC 1	
Remission status prior to HSCT (%)				
• CR 1	6 (20,7%)	3	2	1
• CR ≥2	2 (6,9%)	0	1	1
• PR/VGPR 1	9 (31%)	2	7	0
• PR/VGPR ≥2	6 (20,7%)	4	2	0
• NR	6 (20,7%)	5	1	0
HSCT, depletion technique (%)				
• CD3+/CD19+	20 (69%)	11	7	2
• TCRα/β+ / CD19+	9 (31%)	3	6	0

combination with melphalan (140 mg/m²) and thiotepa (10 mg/kg). Two patients with previous haplo-HSCT and graft failure received additional TLI (4 Gy respectively 7 Gy) to reduce the risk of another graft failure.

Serotherapy was part of the conditioning regimen in all patients, comprising OKT3 (given day -9 until day +17) until 2010 (n=17) and ATG (given day -11 until day -9) since OKT3 was no longer available in 2011 (n=12).

Stem cell source and graft manipulation: Mobilized peripheral blood stem cells (PBSC) using granulocyte colony-stimulating factor (Granocyte®, Lenograstim, Chugai Pharma; Neupogen®, Filgrastim, Amgen) were the stem cell sources harvested from haploidentical family donors. Parents served as stem cell donors in 26 transplants, mismatched adult siblings (n=2) and aunt (n=1) in the remaining. The degree of HLA-mismatch (MM) ranged from 2 mismatches in one case, 3 MMs in 4 patients, 4 MMs in 8 patients, and 5 MM in 16 HSCTs. Grafts were processed with CD3+ and CD19+ depletion (n=20) or TCRα/β+ and CD19+ depletion (n=9) respectively, using the automated CliniMACS® system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described earlier (31). The graft composition is specified in Table 2.

Engraftment: Primary engraftment occurred in 27 patients after a median of 10 days (range 7-15), while 2 patients (CD3+/CD19+ n=1, TCRα/β+ / CD19+ depletion n=1) suffered from primary graft failure (6,9%). Both patients could be rescued, using an immunoablative reconditioning regimen, comprising either fludarabin, thiotepa with or without cyclophosphamide, ATG and TLI as described earlier (32), followed by a second haploidentical graft with CD3+/CD19+ or TCRα/β+ / CD19+-depletion respectively from a different donor. Three patients showed a secondary rejection (secondary graft failure 10,3%). Two patients received an autologous backup 29 and 38 days after HSCT respectively. Both were found to be eligible for another haplo-HSCT and were transplanted with a second CD3+/CD19+ depleted graft 7 months after the first one and further follow-up was counted from the second haplo-HSCT. One of these patients suffered another relapse by that time. The third patient with secondary graft failure was rescued with a graft from a syngeneic sibling 85 days after haplo-HSCT since she received a syngeneic HSCT already before haplo-HSCT.

Immune reconstitution: Data were available for 23 patients. In patients, who received a subsequent haploidentical graft following graft failure, the immune phenotype was counted from the day the second graft was infused.

Recovery of CD56+ natural killer (NK) cells was fast, reaching counts of >100cells/µl mean already at day 14. Reconstitution of CD3 + lymphocytes started at day +30, although cell counts of CD3+ T-lymphocytes at day 90 were below 200/µl (172/µl, range 3 – 728) and CD3+CD8+ below 100/µl (87/µl, range 0 – 601) (Figures 1A, B). Reconstitution of CD3+, or CD56+ lymphocytes was not faster in patients without relapse (Figures 1C, D).

Graft-versus-host disease: Mycophenolate mofetil (MMF) was the only GvHD prophylaxis in all patients (standard dosage of 1200 mg/m² in two single dosage). Acute GvHD was overall diagnosed in 17 patients, two of them after receiving post-transplant DLI due to mixed chimerism. Only 5 patients (3-years CI 16,8%) developed clinically relevant GvHD grade II or higher (II° n=1, III° n=3, IV° n=1). Three patients (3-years CI 10,4%) developed cGvHD (limited disease n=1, extended disease n=2) (Figure 2E).

Relapse: Relapse occurred in 20 patients (ES n=12, alveolar RMS n=7, botryoid RMS n=1) at median 112 days (36 days – 1,6 years) post haplo-HSCT, resulting in a cumulative incidence (CI) of 69% after 3 years. Patients, who responded well on pre-transplant treatment (CR or VGPR) suffered significantly less from relapse (3-years CI of relapse 36,8%) than patients with PR (CI 87,0%, p=0,01) or NR (CI 99,5%, p=0,0003) (Figure 2A). While the CI of relapse was lower in patients transplanted in first CR, VGPR or PR, the difference to patients with CR or PR after previous relapse was not statistically significant (58,6% vs. 68,3%; p=0,62) (Figure 2B). Similarly, we didn't find significant difference in CI of relapse by diagnosis between ES and RMS (ES 80,9% vs. RMS 67,7%; p=0,39) (Figure 2C). None of the patients with NRSTS relapsed. However, this group comprises only 2 patients of whom one died from GvHD 33 months after HSCT. There was no significant difference in CI of relapse in patients who developed aGvHD of any grade compared to those who did not experience aGvHD (no aGvHD 74,8% vs. aGvHD °I-IV 66,4%, p=0,59) (Figure 2D).

Viral reactivation: CMV viremia was detected in 4 patients (3 years CI 13,9%) and ADV viremia in 6 patients (3 years CI 20,6%). ADV infection caused 1 death. There was no patient experiencing post-transplant lymphoproliferative disease.

Transplant-related mortality: Three deaths were attributed to transplantation procedure (ADV infection, GvHD with sepsis, intracranial bleeding), resulting in a TRM of 10,5% after 3 years (Figure 2F).

Survival: The 5-year-OS probability was 16,1% (Figure 3A). Details of the five surviving patients are summarized in Table 3.

The EFS probability was 18,1% after 3 years (Figure 3A) and dependent only on pre-transplant remission status. Patients with at least VGPR experienced lesser events than patients with only partial or worse response (EFS of patients in CR or VGPR (36,4%) vs. PR (9,4%; p=0,08) or NR (0%; p=0,01) (Figure 3B). Patients with first partial or better response fare better than patients who already

TABLE 2 Graft Composition.

	CD34+ *10⁶/kg	CD3+ § *10³/kg	TCRα/β+ ≠ *10³/kg	TCRγ/δ+ ≠ *10⁶/kg	CD19+ *10³/kg	CD56+ *10⁶/kg
minimum	2,57	9,5	8,4	1,5	8,508	23,42
median	11,2	41,33	12,48	9,13	36,12	56,78
maximum	26,13	100,02	35,81	20,85	561,031	298,26
number of patients	29	20	9	9	23	23

In case of reconditioning 1st graft considered. § only CD3+/CD19+ depleted grafts considered. ≠ only TCRα/β+ / CD19+ depleted grafts considered.

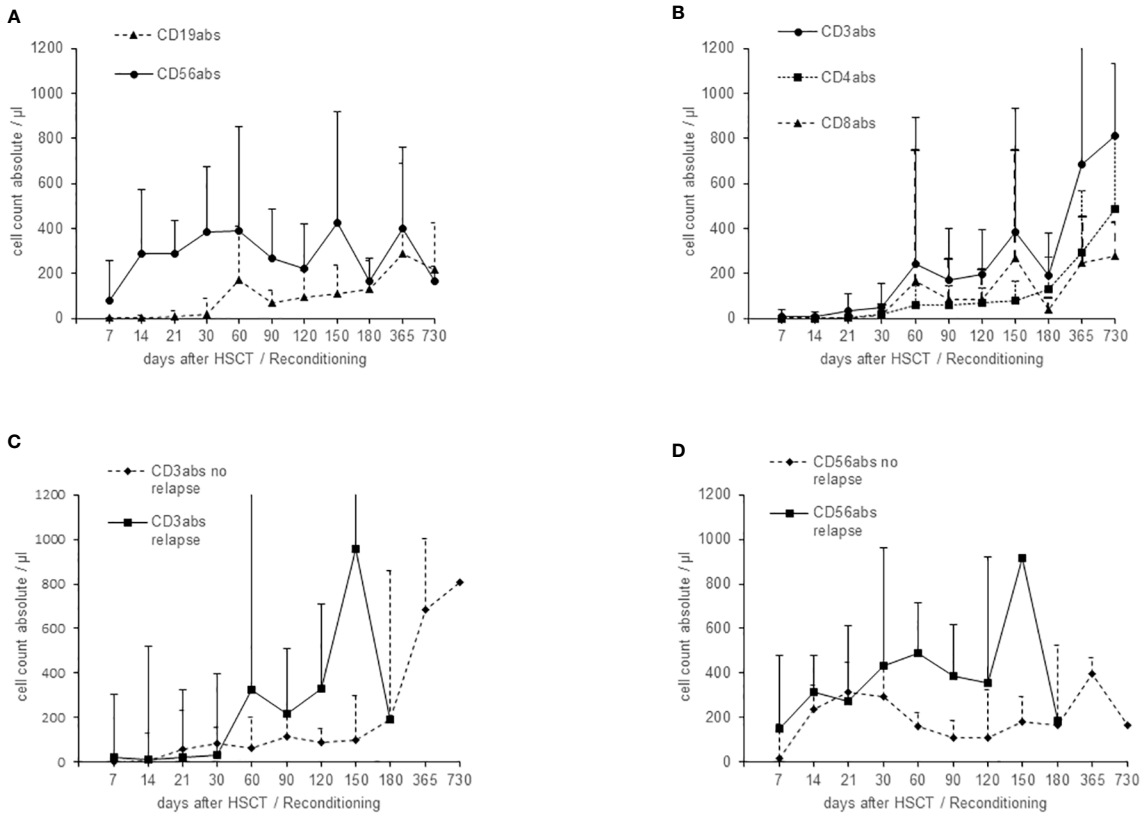


FIGURE 1
Immune reconstitution after haploidentical HSCT (mean and standard deviation), calculated from total lymphocyte cell count and flow cytometry results. **(A)** Reconstitution of CD56+ NK and CD19+ B cells. **(B)** Reconstitution of CD3+ T cells (CD4+ and CD8+). **(C)** Comparison of the recovery of CD3+ T cells in patients with or without relapse. **(D)** Comparison of the recovery of CD56+ NK cells in patients with or without relapse.

experienced relapse pre-transplant. The difference did not reach statistical significance. None of the patients with previous relapse survived regardless of response to pre-transplant treatment. (EFS of patients in first CR, VGPR or PR 33,3% vs. subsequent CR or PR 0%; p=0,2) (Figure 3C). The occurrence of aGvHD did not significantly influence EFS (EFS without aGvHD 9,3% vs. aGvHD I-IV° 23,5%; p=0,45) (Figure 3D). Whether cGvHD developed or not did not affect EFS, whereby patients might have experienced relapse before cGvHD could have developed. The major events were relapse in 19 patients (82%) followed by transplant-related deaths (n=3). In one patient a secondary neoplasm occurred (vulvar intraepithelial neoplasm) 14 months after haplo-HSCT, before relapse of the primary Ewing sarcoma.

Discussion

Haplo-HSCT with selective T-cell depletion has been used in hematological malignancies with impressive results (33) and it is also in part reported in some patients with solid tumors (5, 27). These approaches could have the advantages of providing high doses of cell subsets that mediate the anti-tumor effect through HLA independent pathways without carrying a graft-versus-host effect, such as NK cells that prevail in CD3+-negative selected graft as in the early stages after engraftment. In the more recent TCRα/β+-negative selection, the TCRγ/δ+ lymphocytes, preserved in the graft, further enhance the GvT effect as well as carry out a fundamental antiviral activity (34). Moreover, since in this context prolonged immunosuppression after transplant is not required, the donor-derived T cells activity, whose reconstitution occurs later, is not hindered.

However, while T-cell depleted haploidentical HSCT was safe, in this study it was applicable for patients with response but not effective for patients with advanced pediatric sarcomas not in response after initial therapy.

These results have to be interpreted in the context of the extremely high-risk profile of our cohort. Almost half of our patients (48,3%) experienced one or subsequent metastatic relapses pre-transplant, which occurred early within 2 years after primary diagnosis in 10 patients, especially with ES. More than 20% of patients underwent transplantation in non-response, and another 12 patients (41%) responded just partially before HSCT and have been transplanted with significant residual tumor mass, with consequent increased risk of later relapse or progression. Only 38% of patients achieved CR or VGPR prior to HSCT. The influence of pre-transplant remission status on survival has been reported earlier in patients with Ewing sarcoma (29). Indeed, in our cohort, patients who underwent haplo-HSCT in presence of better remission status (CR or VGPR) showed a significantly lower cumulative incidence of relapse (CIR) and a better EFS than those with PR (CIR 36,8% vs 87,0%, EFS 36,4% vs 9,4%) or persistent disease (CIR 99,5%, EFS 0%) (Figures 2A, 3B). This points up the fundamental role of pre-transplant approaches in pediatric sarcoma, as reported in other malignancies (35). A total of 5

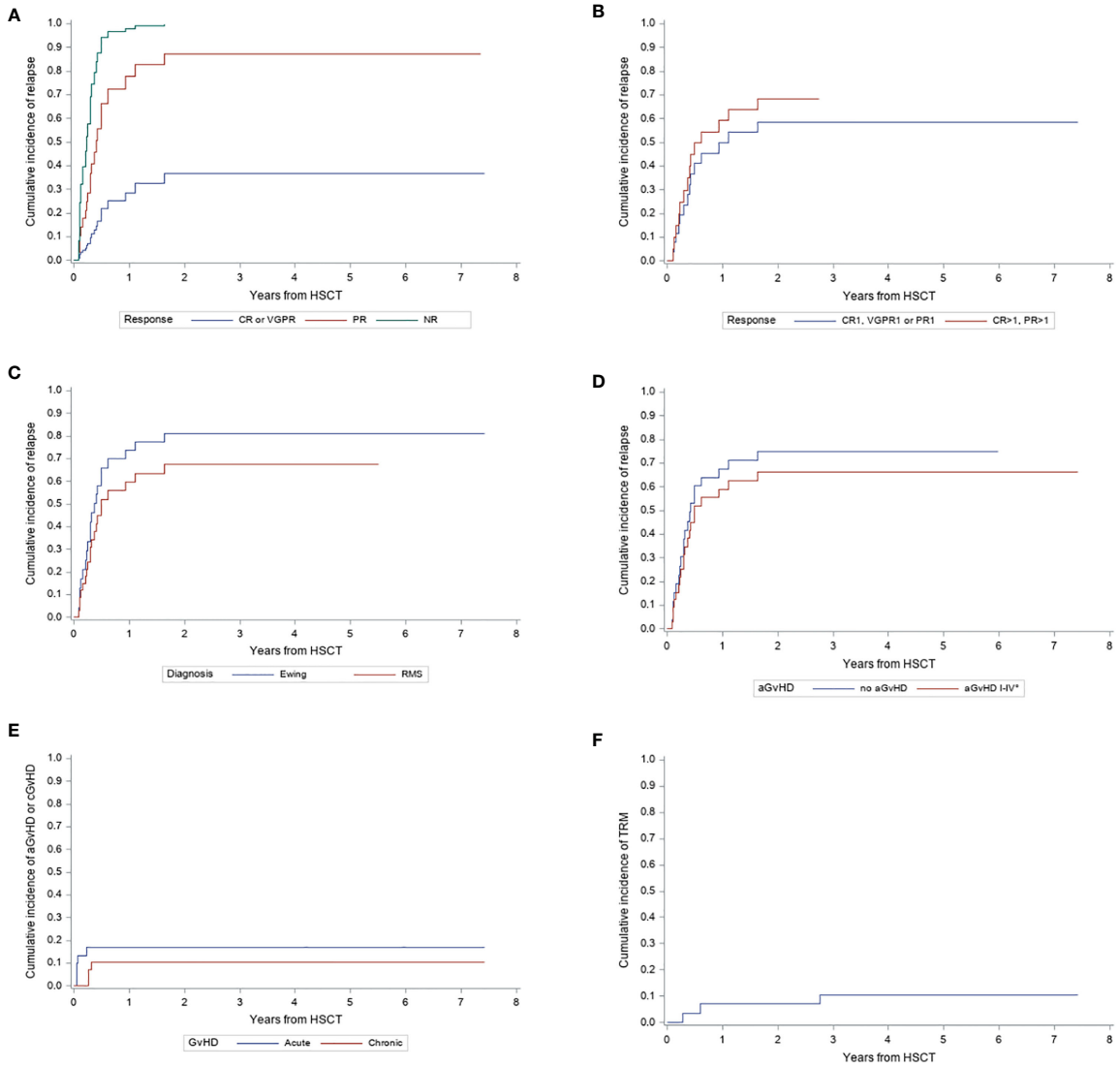


FIGURE 2
Cumulative Incidence (CI) of relapse, GvHD and transplant related mortality from haploidentical HSCT. **(A)** CI of relapse in patients who reach CR or VGPR after pre-transplant therapy compared to patients with either PR or NR. **(B)** CI of relapse in patients achieving first CR, VGPR or PR pre-transplant compared to patients in CR or PR who were transplanted after relapse. **(C)** CI of relapse in patients with Ewing Sarcoma or Rhabdomyosarcoma. **(D)** CI of relapse according to occurrence of aGvHD °I-IV or no aGvHD. **(E)** CI of aGvHD °II-IV and cGvHD. **(F)** CI of transplant related mortality.

patients survived without signs of disease. All of them received a haplo-HSCT in CR1, VGPR1 or PR 1, whereas patients transplanted after relapse could not be rescued. This raises the question if a haplo-HSCT could be a treatment option as consolidation therapy following standard treatment protocols for primary disease, especially in patients with complete or very good partial response or if low dose maintenance chemotherapy will be equal or more effective. Carli et al. reported an EFS of 23% in patients reaching CR after conventional therapy with a prolonged course of chemotherapy in RMS (6). Otherwise, Klingebiel et al. reported an OS of 52% in patients with metastatic soft tissue sarcomas receiving oral maintenance therapy (36). Another matched pair analysis showed no benefit of allogeneic HSCT over non transplanted controls (37) and Merker et al. (38) found no benefit of haplo-HSCT compared to reported results of oral maintenance therapy. However, randomized studies addressing this issue are missing. To achieve statistical relevant patient numbers, a European or even international study would be necessary, although, the feasibility of a randomized study is questionable because of the poor prognosis in this cohort of patients.

Relapse occurred early, within a median of 112 days post-HSCT and thus before sufficient reconstitution of T-cells, probably making too short the timing interval for their contribution to the GvT-effect (Figure 1B). Choosing a more intense conditioning regimen might have prevented early relapse (39) and thus given time for a T-cell mediated GvT effect to fully develop. However, as most of our patients did not respond completely to intense pre-transplant chemotherapy regimen, we regard this effect limited. Furthermore, therapy related toxicity is likely to cause a higher TRM rate in this heavily pretreated patient group and outweigh any benefit from a more intense conditioning.

Despite NK-cells being recovered within two weeks after HSCT, confirming previous observations in T-depleted haplo-HSCT for both malignant (40, 41) and non-malignant diseases (34, 42), and the immunosuppression only being short-term, GvT-effect apparently

10.3389/fonc.2023.1064190

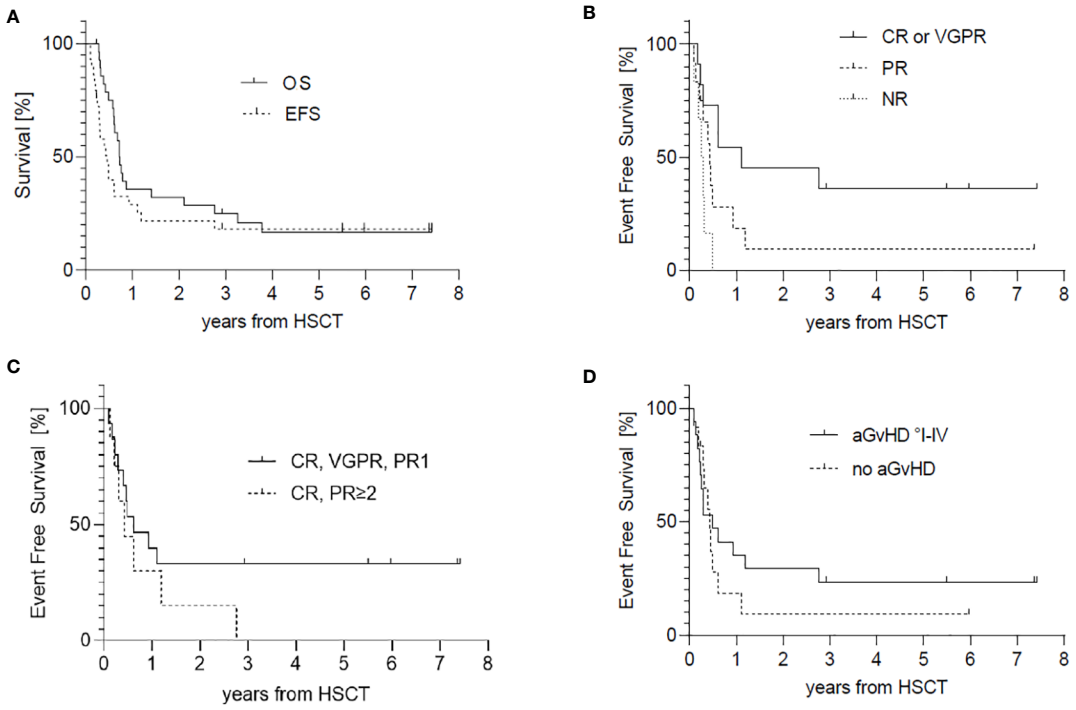


FIGURE 3
Survival, counted from haploidentical HSCT. **(A)** Probability of Overall Survival (OS) and Event Free Survival of the entire cohort. **(B)** EFS according to pre-transplant remission status CR/VGPR vs. PR or NR. **(C)** EFS of patients achieving first PR or better compared to patients in CR/PR, who were transplanted after relapse. **(D)** EFS in accordance with the occurrence of aGvHD.

was not sufficient for the majority of patients in our cohort of pediatric sarcomas. GvHD prophylaxis using MMF was given over a short period of 30d median. Since NK-cell recovery was fast, MMF seems not to have impaired NK-cell proliferation but it cannot be excluded that MMF might have impaired NK-cell function (43) and thus possible GvT- effects in our setting. Beside this, a retrospective analysis by Thiel et al. also could not show a clear GvT-effect in most patients with Ewing sarcoma neither in HLA-mismatched nor in HLA-matched HSCT (29).

Apart from NK-cells, a GvT-effect is often attributed to donor T-cells. Childs et al. reported a regression of metastatic solid tumors after establishment of a complete T-cell chimerism and withdrawal of

TABLE 3 Details of surviving patients.

	Patient I	Patient II	Patient III	Patient IV	Patient V
Diagnosis	Ewing sarcoma	aRMS	aRMS	Undifferentiated sarcoma	Ewing sarcoma
Stage at diagnosis	Stage IV Multiple bone metastases	Stage IV Multiple bone metastases	Stage IV Multiple bone & pelvic & nodal metastases	Stage IV Nodal & pulmonal metastases	Stage IV Multiple bone & nodal metastases
Pre-Transplant Remission status	CR1	VGPR1	VGPR1	CR1	PR1
Conditioning regimen	Treosulfan, Fludarabin, Thiotepa, OKT3	Melphalan, Fludarabin, Thiotepa, ATG	Melphalan, Fludarabin, Thiotepa, ATG, TLI	Melphalan, Fludarabin, Thiotepa, OKT3	Melphalan, Fludarabin, Thiotepa, ATG
Form of graft manipulation	CD3+/CD19+ depletion	CD3+/CD19+ depletion	CD3+/CD19+ depletion	CD3+/CD19+ depletion	TCRα/β+/CD19+ depletion
Donor No of Mismatch	Father 4/10	Father 5/10	Mother 5/10	Mother 5/10	Father 4/10
Chimerism at d100 either CD3, PBMC	Complete	Complete	Complete	Complete	Complete
aGvHD, max. grade	Grade 1	Grade 1	Grade 1	No aGvHD	Grade 3
cGvHD	No cGvHD	No cGvHD	No cGvHD	No cGvHD	No cGvHD
Last follow up in years after HSCT	7,4	5,5	2,9	5,97	7,4

immunosuppression or infusion of donor lymphocytes (17). Since T-cell reconstitution takes time following T-cell depletion, full GvT-effect is delayed as well. Previous reports on haplo-HSCT in pediatric sarcomas based mainly on T-cell depletion (5, 24, 29, 38). An alternative to T-cell depletion in the setting of haplo-HSCT could be the use of T-cell-repleted grafts. However, the use of unmanipulated grafts in haplo-HSCT requires an intense GvHD-prophylaxes (post-transplantation cyclophosphamide or Beijing protocol). Haplo-HSCT using T-cell replete grafts constitutes an interesting approach, however, it has to be determined if the use T-cell-repleted grafts results in a stronger GvT-effect. So far none of the different transplantation protocols was superior over the others regarding OS or relapse (44).

This work has limitations and strengths. The main limitations consist of the retrospective analysis and the small number and heterogeneity of diagnosis of patients included. Furthermore, the inclusion of patients with no response to initial therapy can be questioned. However, this study offers the opportunity to address important questions about haplo-SCT in a subgroup of pediatric solid tumors with poor prognosis.

Based on the above-mentioned results, haploidentical HSCT seems not to considerably improve outcome in most patients with high-risk Ewing sarcoma and RMS, particularly without good response on initial treatment. This observation corresponds with our experience in relapsed neuroblastoma (45). Nevertheless, dose-escalation of conventional therapy including high-dose chemotherapy reached its limits without substantial improvement in survival (15). Therefore, targeted therapies and immunotherapies remain an area of special interest in pediatric sarcomas, as for other cancers. There are hints, that IL2 given posttransplant can augment donor derived NK cell activity in a rather unspecific way (26, 46). On the other hand, another study about prophylactic IL2 administration found an increased risk of relapse (47). Thus, more specific immunotherapies are needed. Based on the experience in leukemias and neuroblastomas, treatment with appropriate monoclonal antibodies (mAb) or antibody-cytokine fusion proteins post-transplant, chimeric antigen receptor (CAR)-T-cell therapy and more recently CAR-NK-cells from the stem cell donor might be potential therapeutic approaches in the future (48). The identification of a tumor-antigen with features of high expression on tumor cells and low expression on healthy tissues remains the main challenge for an effective and safe clinical translation of such approaches. Different possible targets for CAR T-cells or mAbs are currently under investigation in sarcomas, including HER2 (49, 50), B7-H3 (51), ErbB2 (52), GD2 (53, 54), or VEGFR2 (55). The possibility to apply an antibody, CAR-T or CAR-NK approach based on the donor derived immune system in a haploidentical context could represent a fascinating option for the future.

The high safety profile of haploidentical HSCT with CD3+/CD19+ (56) or TCRαβ+/CD19+ depletion (28), carrying on low GvHD-rate and TRM, makes it feasible even in heavily pretreated patients, representing an ideal basis for potential subsequent immunotherapies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

PL and TE conceived the idea of this work. PL, RH and EK designed the clinical trials in which the reported patients participated. PL, RH, MA, H-MT, MM, UH, ME, WS, TF, CS, BG, MD and TE contributed to the acquisition and analysis of data. TE, MD and SG drafted the manuscript with the help of the other authors. PL, MS-S, ME, EK, and TF critically revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the excellence cluster iFIT (EXC 2180) [Gefördert durch die Deutsche Forschungsgemeinschaft (DFG) im Rahmen der Exzellenzstrategie des Bundes und der Länder – WXC 2180 – 390900677], from the German Cancer Consortium (DKTK) and from the Dieter Schwarz Stiftung Neckarsulm to P.L. We thank the Foerderverein and the Stiftung fuer krebskranke Kinder Tuebingen e.V. for continuous support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Erdmann F, Kaatsch P, Grabow D, Spix CGerman Childhood Cancer Registry - Annual Report 2019 (1980-2018). *Institute of medical biostatistics, epidemiology and informatics (IMBEI) at the university medical center of the Johannes Gutenberg university mainz*. (2020). Deutsches Kinderkrebsregister, Mainz

2. Breneman JC, Lyden E, Pappo AS, Link MP, Anderson JR, Parham DM, et al. Prognostic factors and clinical outcomes in children and adolescents with metastatic rhabdomyosarcoma–a report from the intergroup rhabdomyosarcoma study IV. *J Clin Oncol* (2003) 21(1):78–84. doi: 10.1200/JCO.2003.06.129

3. Koscielniak E, Klingebiel TH, Peters C, Hermann J, Burdach ST, Bender-Gotze C, et al. Do patients with metastatic and recurrent rhabdomyosarcoma benefit from high-dose therapy with hematopoietic rescue? Report of the German/Austrian pediatric bone marrow transplantation group. *Bone Marrow Transplant* (1997) 19(3):227–31. doi: 10.1038/sj.bmt.1700628

4. Pappo AS, Anderson JR, Crist WM, Wharam MD, Breitfeld PP, Hawkins D, et al. Survival after relapse in children and adolescents with rhabdomyosarcoma: A report from the intergroup rhabdomyosarcoma study group. *J Clin Oncol* (1999) 17(11):3487–93. doi: 10.1200/JCO.1999.17.11.3487

5. Koscielniak E, Gross-Wieltsch U, Treuner J, Winkler P, Klingebiel T, Lang P, et al. Graft-versus-Ewing sarcoma effect and long-term remission induced by haploidentical stem-cell transplantation in a patient with relapse of metastatic disease. *J Clin Oncol* (2005) 23(1):242–4. doi: 10.1200/JCO.2005.05.940

6. Carli M, Colombatti R, Oberlin O, Bisogno G, Treuner J, Koscielniak E, et al. European Intergroup studies (MMT4-89 and MMT4-91) on childhood metastatic rhabdomyosarcoma: final results and analysis of prognostic factors. *J Clin Oncol* (2004) 22(23):4787–94. doi: 10.1200/JCO.2004.04.083

7. Oberlin O, Rey A, Lyden E, Bisogno G, Stevens MC, Meyer WH, et al. Prognostic factors in metastatic rhabdomyosarcomas: Results of a pooled analysis from united states and European cooperative groups. *J Clin Oncol* (2008) 26(14):2384–9. doi: 10.1200/JCO.2007.14.7207

8. Thiel U, Wawer A, Wolf P, Badoglio M, Santucci A, Klingebiel T, et al. No improvement of survival with reduced- versus high-intensity conditioning for allogeneic stem cell transplants in Ewing tumor patients. *Ann Oncol* (2011) 22(7):1614–21. doi: 10.1093/annonc/mdq703

9. Ladenstein R, Potschger U, Le Deley MC, Whelan J, Paulussen M, Oberlin O, et al. Primary disseminated multifocal Ewing sarcoma: Results of the Euro-EWING 99 trial. *J Clin Oncol* (2010) 28(20):3284–91. doi: 10.1200/JCO.2009.22.9864

10. Cotterill SJ, Ahrens S, Paulussen M, Jurgens HF, Voute PA, Gadner H, et al. Prognostic factors in ewing's tumor of bone: Analysis of 975 patients from the European intergroup cooperative ewing's sarcoma study group. *J Clin Oncol* (2000) 18(17):3108–14. doi: 10.1200/JCO.2000.18.17.3108

11. Brennan B, Stevens M, Kelsey A, Stiller CA. Synovial sarcoma in childhood and adolescence: a retrospective series of 77 patients registered by the children's cancer and leukaemia group between 1991 and 2006. *Pediatr Blood Cancer* (2010) 55(1):85–90. doi: 10.1002/pbc.22453

12. Scheer M, Dantonello T, Hallmen E, Blank B, Sparber-Sauer M, Vokuhl C, et al. Synovial sarcoma recurrence in children and young adults. *Ann Surg Oncol* (2016) 23 (Suppl 5):618–26. doi: 10.1245/s10434-016-5535-2

13. Ferrari A, De Salvo GL, Dall'Igna P, Meazza C, De Leonardis F, Manzitti C, et al. Salvage rates and prognostic factors after relapse in children and adolescents with initially localised synovial sarcoma. *Eur J Cancer* (2012) 48(18):3448–55. doi: 10.1016/j.ejca.2012.06.017

14. Whelan J, Le Deley MC, Dirksen U, Le Teuff G, Brennan B, Gaspar N, et al. High-dose chemotherapy and blood autologous stem-cell rescue compared with standard chemotherapy in localized high-risk Ewing sarcoma: Results of Euro-E.W.I.N.G.99 and Ewing-2008. *J Clin Oncol* (2018) 36(31):JCO2018782516. doi: 10.1200/JCO.2018.78.2516

15. Barrett D, Fish JD, Grupp SA. Autologous and allogeneic cellular therapies for high-risk pediatric solid tumors. *Pediatr Clin North Am* (2010) 57(1):47–66. doi: 10.1016/j.pcl.2010.01.001

16. Meyers PA, Krailo MD, Ladanyi M, Chan KW, Sailer SL, Dickman PS, et al. High-dose melphalan, etoposide, total-body irradiation, and autologous stem-cell reconstitution as consolidation therapy for high-risk ewing's sarcoma does not improve prognosis. *J Clin Oncol* (2001) 19(11):2812–20. doi: 10.1200/JCO.2001.19.11.2812

17. Childs R, Chernoff A, Contentin N, Bahceci E, Schrump D, Leitman S, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* (2000) 343(11):750–8. doi: 10.1056/NEJM200009143431101

18. Lundqvist A, Childs R. Allogeneic hematopoietic cell transplantation as immunotherapy for solid tumors: Current status and future directions. *J Immunother* (2005) 28(4):281–8. doi: 10.1097/01.cji.0000165354.19171.8f

19. Ohta H, Hashii Y, Yoshida H, Kusuki S, Tokimasa S, Yoneda A, et al. Allogeneic hematopoietic stem cell transplantation against recurrent rhabdomyosarcoma. *J Pediatr Hematol Oncol* (2011) 33(1):e35–8. doi: 10.1097/MPH.0b013e3181e7ddc5

20. Yamazaki F, Osumi T, Shigematsu N, Morioka H, Shimada H. Successful treatment of metastatic rhabdomyosarcoma with radiochemotherapy and allogeneic hematopoietic stem cell transplantation. *Jpn J Clin Oncol* (2015) 45(2):225–8. doi: 10.1093/jjco/hyu189

21. Misawa A, Hosoi H, Tsuchiya K, Iehara T, Sawada T, Sugimoto T. Regression of refractory rhabdomyosarcoma after allogeneic stem-cell transplantation. *Pediatr Hematol Oncol* (2003) 20(2):151–5. doi: 10.1080/0880010390158658

22. Lucas KG, Schwartz C, Kaplan J. Allogeneic stem cell transplantation in a patient with relapsed Ewing sarcoma. *Pediatr Blood Cancer* (2008) 51(1):142–4. doi: 10.1002/pbc.21503

23. Donker AE, Hoogerbrugge PM, Mavinkurve-Groothuis AM, van de Kar NC, Boetes C, Hulsbergen-van de Kaa CA, et al. Metastatic rhabdomyosarcoma cured after chemotherapy and allogeneic SCT. *Bone Marrow Transplant* (2009) 43(2):179–80. doi: 10.1038/bmt.2008.301

24. Pession A, Masetti R, Di Leo C, Franzoni M, Prete A. HLA-mismatched hematopoietic stem cell tranplantation for pediatric solid tumors. *Pediatr Rep* (2011) 3 Suppl 2(Suppl 2):e12. doi: 10.4081/pr.2011.s2.e12

25. Baird K, Fry TJ, Steinberg SM, Bishop MR, Fowler DH, Delbrook CP, et al. Reduced-intensity allogeneic stem cell transplantation in children and young adults with ultrahigh-risk pediatric sarcomas. *Biol Blood Marrow Transplant* (2012) 18(5):698–707. doi: 10.1016/j.bbmt.2011.08.020

26. Lang P, Pfeiffer M, Muller I, Schumm M, Ebinger M, Koscielniak E, et al. Haploidentical stem cell transplantation in patients with pediatric solid tumors: preliminary results of a pilot study and analysis of graft versus tumor effects. *Klin Padiatr* (2006) 218(6):321–6. doi: 10.1055/s-2006-942256

27. Schlegel P, Feuchtinger T, Nitschke-Gerard C, Seidel UJ, Lang AM, Kyzirakos C, et al. Favorable NK cell activity after haploidentical hematopoietic stem cell transplantation in stage IV relapsed ewing's sarcoma patients. *Bone Marrow Transplant* (2015) 50 Suppl 2:S72–6. doi: 10.1038/bmt.2015.100

28. Bethge WA, Eyrich M, Mielke S, Meisel R, Niederwieser D, Schlegel PG, et al. Results of a multicenter phase I/II trial of TCRalphabeta and CD19-depleted haploidentical hematopoietic stem cell transplantation for adult and pediatric patients. *Bone Marrow Transplant* (2022) 57(3):423–30. doi: 10.1038/s41409-021-01551-z

29. Thiel U, Schober SJ, Ranft A, Gassmann H, Jabar S, Gall K, et al. No difference in survival after HLA mismatched versus HLA matched allogeneic stem cell transplantation in Ewing sarcoma patients with advanced disease. *Bone Marrow Transplant* (2021) 56 (7):1550–7. doi: 10.1038/s41409-020-01200-x

30. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-a-matched sibling donors. *Transplantation* (1974) 18(4):295–304. doi: 10.1097/00007890-197410000-00001

31. Schumm M, Handgretinger R, Pfeiffer M, Feuchtinger T, Kuci S, Faul C, et al. Determination of residual T- and b-cell content after immunomagnetic depletion: proposal for flow cytometric analysis and results from 103 separations. *Cytotherapy* (2006) 8(5):465–72. doi: 10.1080/14653240600932787

32. Lang P, Mueller I, Greil J, Bader P, Schumm M, Pfeiffer M, et al. Retransplantation with stem cells from mismatched related donors after graft rejection in pediatric patients. *Blood Cells Mol Dis* (2008) 40(1):33–9. doi: 10.1016/j.bcmd.2007.06.027

33. Gonzalez-Vicent M, Diaz Perez MA. Allogeneic hematopoietic stem-cell transplantation from haploidentical donors using 'ex-vivo' T-cell depletion in pediatric patients with hematological malignancies: State of the art review. *Curr Opin Oncol* (2018) 30(6):396–401. doi: 10.1097/CCO.0000000000000480

34. Giardino S, Bagnasco F, Falco M, Miano M, Pierri F, Risso M, et al. Haploidentical stem cell transplantation after TCR-alphabeta(+) and CD19(+) cells depletion in children with congenital non-malignant disease. *Transplant Cell Ther* (2022) 28(7):394 e1– e9. doi: 10.1016/j.jtct.2022.04.002

35. Bader P, Hancock J, Kreyenberg H, Goulden NJ, Niethammer D, Oakhill A, et al. Minimal residual disease (MRD) status prior to allogeneic stem cell transplantation is a powerful predictor for post-transplant outcome in children with ALL. *Leukemia* (2002) 16 (9):1668–72. doi: 10.1038/sj.leu.2402552

36. Klingebiel T, Boos J, Beske F, Hallmen E, Int-Veen C, Dantonello T, et al. Treatment of children with metastatic soft tissue sarcoma with oral maintenance compared to high dose chemotherapy: Report of the HD CWS-96 trial. *Pediatr Blood Cancer* (2008) 50(4):739–45. doi: 10.1002/pbc.21494

37. Schober SJ, Hallmen E, Ressle F, Gassmann H, Prexler C, Wawer A, et al. No improvement of survival for alveolar rhabdomyosarcoma patients after HLA-matched versus -mismatched allogeneic hematopoietic stem cell transplantation compared to standard-of-Care therapy. *Front Oncol* (2022) 12:878367. doi: 10.3389/fonc.2022.878367

38. Merker M, Meister MT, Rettinger E, Jarisch A, Soerensen J, Willasch A, et al. Haploidentical allogeneic hematopoietic stem cell transplantation in patients with high-risk soft tissue sarcomas: Results of a single-center prospective trial. *Bone Marrow Transplant* (2018) 53(7):891–4. doi: 10.1038/s41409-018-0088-6

39. Dillon LW, Gui G, Logan BR, Fei M, Ghannam J, Li Y, et al. Impact of conditioning intensity and genomics on relapse after allogeneic transplantation for patients with myelodysplastic syndrome. *JCO Precis Oncol* (2021) 5, 265–274. doi: 10.1200/PO.20.00355

40. Maschan M, Shelikhova L, Ilushina M, Kurnikova E, Boyakova E, Balashov D, et al. TCR-alpha/beta and CD19 depletion and treosulfan-based conditioning regimen in unrelated and haploidentical transplantation in children with acute myeloid leukemia. *Bone Marrow Transplant* (2016) 51(5):668–74. doi: 10.1038/bmt.2015.343

41. Locatelli F, Merli P, Pagliara D, Li Pira G, Falco M, Pende D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after alphabeta T-cell and b-cell depletion. *Blood* (2017) 130(5):677–85. doi: 10.1182/blood-2017-04-779769

42. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-haploidentical stem cell transplantation after removal of alphabeta+ T and b cells in children with nonmalignant disorders. *Blood* (2014) 124(5):822–6. doi: 10.1182/blood-2014-03-563817

43. Yu XX, Cao XH, Yan H, Luo XY, Zhao XS, Sun YQ, et al. Delay expression of NKp30 on NK cells correlates with long-term mycophenolate mofetil treatment and higher EBV viremia post allogenic hematological stem cells transplantation. *Clin Immunol* (2019) 205:49–56. doi: 10.1016/j.clim.2019.05.010

44. Kleinschmidt K, Lv M, Yanir A, Palma J, Lang P, Eyrich M. T-Cell-Replete versus ex vivo T-Cell-Depleted haploidentical haematopoietic stem cell transplantation in children with acute lymphoblastic leukaemia and other haematological malignancies. *Front Pediatr* (2021) 9:794541. doi: 10.3389/fped.2021.794541

45. Illhardt T, Toporski J, Feuchtinger T, Turkiewicz D, Teltschik HM, Ebinger M, et al. Haploidentical stem cell transplantation for Refractory/Relapsed neuroblastoma. *Biol Blood Marrow Transplant* (2018) 24(5):1005–12. doi: 10.1016/j.bbmt.2017.12.805

46. Schlegel P, Teltschik HM, Pfeiffer M, Handgretinger R, Schumm M, Koscielniak E, et al. Long-term IL-2 therapy after transplantation of T cell depleted stem cells from alternative donors in children. *Best Pract Res Clin Haematol* (2011) 24(3):443–52. doi: 10.1016/j.beha.2011.04.007

47. Zhao XY, Zhao XS, Wang YT, Chen YH, Xu LP, Zhang XH, et al. Prophylactic use of low-dose interleukin-2 and the clinical outcomes of hematopoietic stem cell transplantation: A randomized study. *Oncoimmunology* (2016) 5(12):e1250992. doi: 10.1080/2162402X.2016.1250992

48. Roberts SS, Chou AJ, Cheung NK. Immunotherapy of childhood sarcomas. *Front Oncol* (2015) 5:181. doi: 10.3389/fonc.2015.00181

49. *Her2 chimeric antigen receptor expressing T cells in advanced sarcoma* (2021). Available at: https://clinicaltrials.gov/ct2/show/NCT00902044 (Accessed September 27,2022). ClinicalTrials.gov Identifier.

50. Hegde M, Joseph SK, Pashankar F, DeRenzo C, Sanber K, Navai S, et al. Tumor response and endogenous immune reactivity after administration of HER2 CAR T cells in a child with metastatic rhabdomyosarcoma. *Nat Commun* (2020) 11(1):3549. doi: 10.1038/s41467-020-17175-8

51. Majzner RG, Theruvath JL, Nellan A, Heitzeneder S, Cui Y, Mount CW, et al. CAR T cells targeting B7-H3, a pan-cancer antigen, demonstrate potent preclinical activity against pediatric solid tumors and brain tumors. *Clin Cancer Res* (2019) 25(8):2560–74. doi: 10.1158/1078-0432.CCR-18-0432

52. Merker M, Wagner J, Kreyenberg H, Heim C, Moser LM, Wels WS, et al. ERBB2-CAR-Engineered cytokine-induced killer cells exhibit both CAR-mediated and innate immunity against high-risk rhabdomyosarcoma. *Front Immunol* (2020) 11:581468. doi: 10.3389/fimmu.2020.581468

53. Golinelli G, Grisendi G, Dall'Ora M, Casari G, Spano C, Talami R, et al. Anti-GD2 CAR MSCs against metastatic ewing's sarcoma. *Transl Oncol* (2022) 15(1):101240. doi: 10.1016/j.tranon.2021.101240

54. *A phase I trial of T cells expressing an anti-GD2 chimeric antigen receptor in children and young adults with GD2+ solid tumors* (2022) (Accessed September 27,2022). ClinicalTrials.gov Identifier.

55. Englisch A, Altvater B, Kailayangiri S, Hartmann W, Rossig C. VEGFR2 as a target for CAR T cell therapy of Ewing sarcoma. *Pediatr Blood Cancer* (2020) 67(10):e28313. doi: 10.1002/pbc.28313

56. Lang P, Teltschik HM, Feuchtinger T, Muller I, Pfeiffer M, Schumm M, et al. Transplantation of CD3/CD19 depleted allografts from haploidentical family donors in paediatric leukaemia. *Br J Haematol* (2014) 165(5):688–98. doi: 10.1111/bjh.12810

Frontiers in Oncology

Advances knowledge of carcinogenesis and tumor progression for better treatment and management

The third most-cited oncology journal, which highlights research in carcinogenesis and tumor progression, bridging the gap between basic research and applications to imrpove diagnosis, therapeutics and management strategies.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact