NEW INSIGHTS IN THE LANDSCAPE OF RARE TUMORS: TRANSLATIONAL AND CLINICAL RESEARCH PERSPECTIVE

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NEW INSIGHTS IN THE LANDSCAPE OF RARE TUMORS: TRANSLATIONAL AND CLINICAL RESEARCH PERSPECTIVE

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EDITORIAL
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Editorial: New Insights Into the Landscape of Rare Tumors: Translational and Clinical Research Perspective

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Editorial on the Research Topic

New Insights Into the Landscape of Rare Tumors: Translational and Clinical Research Perspective

In the landscape of solid and liquid malignancies, rare tumors represent a major challenge for patient management in terms of their biology, classification, clinical behavior (1). In this scenario, the term *rare tumor* refers to a multitude of heterogeneous diseases often characterized by diagnostic pitfalls, the unavailability of prognostic and predictive biomarkers, and the lack of standardized treatments (2). Such issues are reflected in clinical outcome, which is generally worse than that of patients with more common tumors (3). Consequently, there is a pressing need for a better understanding of the natural history of these diseases and for the development of innovative treatment strategies (4, 5). Building a strong collaborative network between physicians, researchers, and institutions will be key to achieving the above goals (6).

The Research Topic of this special issue takes an in-depth look at recent translational and clinical advancements in the area of rare tumors. A collection of original articles, systematic reviews, methods, opinions and perspectives will provide readers with news on exciting breakthroughs in research into sporadic rare tumors including bone and soft tissue sarcoma (7, 8), neuroendocrine and endocrine gland neoplasms (9), brain tumors (10), cholangiocarcinoma (11), and rare familial syndromes such von Hippel-Lindau disease (12).

In this regard, the limited availability of biomarkers represents a substantial problem for the management of sarcomas. Furthermore, the unusual histologic features of these malignancies increase the risk of misdiagnosis, leading to the use of an ineffective therapeutic strategy and a poor outcome. Next-generation sequencing (NGS) technology would appear to be a promising tool for sarcoma diagnosis. The identification of histotype-specific gene alterations is of paramount importance for the differential diagnosis of sarcoma variants as almost 30% of sarcomas harbor specific genetic alterations. Racanelli et al. focused on NGS RNA-based approaches to detect sarcoma-specific rearrangements, confirming their potential usefulness in routine diagnostic setting. This is especially important as the identification of a specific genetic alteration may form the basis for a therapeutic option. For example, gastrointestinal stromal tumors (GIST) commonly harbor KIT or PDGFRA mutations and less frequently show SDH or NF1 gene inactivation. Only 10% of GIST are wild type, thus limiting therapeutic opportunities and increasing the risk of poor

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outcome. Astolfi et al. investigated the accuracy of NGS-based second-level molecular analysis for wild type GIST diagnosis. They found that 20% of wild type GIST harbored pathogenic KIT mutations and became eligible for TK inhibitors, underlining the importance of NGS technologies as a diagnostic tool.

The identification of prognostic biomarkers represents another unmet clinical need. Cheng et al. evaluated pretreatment inflammatory indexes as a prognostic predictor of survival in patients with synovial sarcoma, concluding that neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) were independent prognostic factors of progression-free survival (PFS) and overall survival (OS) in this sarcoma histotype. An increasing interest is also being shown in hematological markers as a reliable prognostic tool for sarcoma management. Li et al. discussed the role of prognostic hematological biomarkers in soft tissue sarcoma. In particular, they found that higher NLR, C-reactive protein (CRP), and platelet-to-lymphocyte ratio (PLR) were associated with poor OS/disease-free survival (DFS), whereas a low LMR was linked to worse OS/DFS. Moreover, higher Glasgow prognostic scores (GPS) were correlated with poorer OS/disease-specific survival (DSS). Despite the discovery of genetic aberrations and a deeper understanding of their role in sarcoma pathophysiology, the cornerstone of treatment for the majority of these advanced and metastatic tumors, chemotherapy, has changed little over the years. Thus, the identification of predictive biomarkers to discriminate between responsive and non-responsive patients would represent an important step forward in the management of the disease. Caruso and Garofalo provided an overview of soft tissue sarcoma pharmacogenomic biomarkers currently used to monitor the responsiveness and toxicity of conventional and new chemotherapeutic drugs in soft tissue sarcoma histotypes. For example, extraskeletal myxoid chondrosarcoma is a rare soft tissue sarcoma characterized by an indolent behavior but with an increasing proportion of patients who develop local and distant recurrences. In the latter, standard front-line treatment using anthracycline-based chemotherapy has shown limited activity. Chiusole et al. retrospectively investigated a series of extraskeletal myxoid chondrosarcoma patients, observing that a primary tumor site in the extremities and solitary lung metastases were associated with a better survival. Their results also highlighted a poor performance of anthracycline-based chemotherapy, indicating the need to identify other active treatments.

Similar problems are encountered for the management of bone sarcoma, which also suffers from limited therapeutic options. Despite an aggressive neoadjuvant approach based on cisplatin, doxorubicin, methotrexate and ifosfamide, patients continue to relapse. Fanelli et al. took an in-depth look at cisplatin resistance in osteosarcoma, assessing the value as therapeutic targets of DNA repair-related factors belonging to nucleotide excision repair (NER) or base excision repair (BER) pathways as well as a group kinases. The authors identified NSC130813 (NERI02; F06) and triptolide as valuable agents for overcoming cisplatin resistance, and also confirmed mitogen-activated protein kinase (MAPK) and fibroblast growth factor receptor (FGFR) pathways as novel therapeutic targets in this disease setting.

Another aggressive bone sarcoma and the second most common bone malignancy in young patients is Ewing's sarcoma, characterized by a specific 11:22 chromosomal translocation that generates the EWS/FLI1 fusion oncogene. This malignancy shows rapid growth and early metastasis. Mancarella et al. identified IGF2BP3 as a promising marker for Ewing sarcoma progression and CD164 and CXCR4 as novel IGF2BP3 downstream functional effectors.

In addition to sarcomas, neuroendocrine neoplasms and endocrine gland tumors represent a large group of heterogeneous rare malignancies. These tumors show a wide variety of clinical presentations and although some progress has been made in recent years in terms of diagnosis and pathology classification, their management and treatment remain challenging. The natural history of these tumors is still poorly understood. For example, pancreatic neuroendocrine tumors (pNETs) account for less than 3% of all pancreatic malignancies. Bocchini et al. provided a comprehensive overview of experimental, prognostic and predictive biomarkers available in clinical practice that can be used to facilitate early diagnosis, estimate prognosis and guide the choice of treatment. Their state-of-the-art paper could be a starting point for further research aimed at improving our understanding and clinical management of this complex disease. Recently, preclinical and retrospective clinical data identified a potential anticancer effect mediated by the oral hypoglycemic agent, metformin, in pNETs. Vernieri et al. studied the impact of the drug on the metabolism of pNET patients and its potential role in the treatment of pNETs. The authors also presented a brief overview of current prospective trials investigating the activity of metformin in combination with standard therapies in this disease setting.

The limited availability of systemic therapeutic options represents a hot topic within the context of metastatic neuroendocrine neoplasia (NEN). Bongiovanni et al. carried out a systematic review and meta-analysis of the efficacy and safety of the tyrosine kinase inhibitor (TKI) pazopanib in patients with metastatic and locally advanced NEN. Their results confirmed the efficacy of the drug in these subgroups, the overall response rate comparable with that of other TKIs and mTOR inhibitors, and furnished a rationale to better understand the role of pazopanib in these malignancies.

Von Hippel-Lindau (VHL) disease is a complex inherited disorder characterized by several types of tumors (including pNETs) arising in multiple organs. The clinical effects of TKIs on VHL disease-related tumors are still largely unknown. Ma et al. retrospectively analyzed the response of VHL patients to TKIs, their results suggesting a potential activity of these inhibitors in this disease setting with a manageable toxicity profile.

Among endocrine tumors, thyroid cancer represents the most frequent malignancy and anaplastic thyroid cancer (ATC) the most aggressive histotype. ATC is characterized by limited therapeutic regimens and poor prognosis. Lin et al. investigated the combination of GSK-J4 and doxorubicin in *in*

vitro and *in vivo* anaplastic thyroid cancer models, reporting an activity of the treatment in KRAS-mutant ATC.

Cholangiocarcinoma is a rare and highly fatal malignant tumor of the bile duct, with a poorly understood biological and clinical behavior. Although the tumor has a lower incidence in adolescents and young adults (AYA), this subgroup shows the poorest OS. Feng et al. analyzed three different data sets of AYA cholangiocarcinoma, indentifying ASXL1 and KMT2C as potentially targetable genomic signatures for these patients and providing new insights into this disease.

With regard to the central nervous system, glioblastoma (GBM) represents the most aggressive of all brain tumors. Although prognosis is very poor, a methylated state of the MGMT gene promoter has been shown to predict a better response to temozolomide therapy. Brigliadori et al. identified an intermediate range of methylation (*gray zone*) above the standard cutoff in which the predictive strength of the marker was lost. The authors performed a preliminary assessment on samples belonging to the *gray zone*, confirming the hypothesis of a mismatch between methylation values used for clinical decision making and the variability of the methylation status of each sample. Further research is needed to better define the predictive power of this marker.

An important aspect of the era of multidisciplinary cancer research is that of networking, especially for rare tumors. Melis et al. proposed a network model for clinical and translational research into thymic epithelial tumors. This tool could also be used to implement therapeutic and management facilitate strategies within the more general context of rare tumors.

In conclusion, rare tumors now account for 25% of all cancers. Although these tumors have a low incidence of less than 6 per 100,000 inhabitants, in many cases they have a high prevalence (13), indicating the importance of directing our efforts at promoting interdisciplinary collaborations in care, research and educational areas. Understanding the natural history of these tumors would constitute a substantial breakthrough in preventing, diagnosing earlier and more accurately, and proposing new targeted and interdisciplinary therapeutic approaches.

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OPINION
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Impact of Metformin on Systemic Metabolism and Survival of Patients With Advanced Pancreatic Neuroendocrine Tumors

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INTRODUCTION

Pancreatic neuroendocrine tumors (pNETs) represent a subgroup of neuroendocrine malignancies with specific biological and clinical characteristics, whose incidence has increased in the last four decades. The growth and proliferation of pNET cells is especially dependent on the IGF-1/IGF1-receptor/PI3K/AKT/mTOR signaling pathway, while the activation of somatostatin receptor axis exerts antiproliferative effects [**Figure 1**; (1, 2)].

In recent years, the following clinical achievements dramatically expanded the therapeutic armamentarium against advanced pNETs: (a) somatostatin analogs (SAs) significantly prolonged patient progression free survival (PFS) when compared to the placebo in patients with advanced disease (3); (b) the mTOR inhibitor everolimus and the multi-tyrosine kinase inhibitor sunitinib demonstrated anticancer activity and prolonged median PFS in pNET patients pre-treated with SAs (4, 5); (c) peptide receptor radiotherapy (PRRT) showed impressive anticancer activity coupled with excellent tolerability profiles in patients with pre-treated advanced pNETs. Finally, cytotoxic chemotherapy or liver-directed treatments remain a valid option for patients with high-grade pNETs, as well as for neoplasms progressing on biological agents. Despite these improvements, most advanced pNETs remain almost invariably incurable, and many patients finally die of their disease. Indeed, while 5-year survival is 65–94% in patients with limited-stage disease, it is reduced to 44–76% in the presence of lymph node metastases, and to 27% in the case of distant metastases (6, 7). In the advanced disease setting, factors associated with poorer survival include the presence of liver and peritoneal metastases, not having undergone resection the primary (pancreatic) tumor, high-grade (G3) disease. Therefore, new treatment options are needed for patients with advanced pNETs, in particular those with poor prognostic factors.

In recent years, preclinical and retrospective clinical data have shown that the antidiabetic compound metformin may have antitumor activity against different tumor types including pNETs. Potential mechanisms of metformin anticancer effects include: (1) modifications of systemic metabolism, including a reduction of blood glucose and insulin, which sustain cancer cell growth by fueling cell metabolism; (2) direct, cell-autonomous anticancer effects, which are mediated by the inhibition of mitochondrial metabolism and ATP production, with the consequent impairment of intracellular energetic status and inhibition of mTOR, protein and fatty acid biosynthesis. However, the potential anticancer role of metformin in patients with advanced pNETs remains to be fully elucidated.

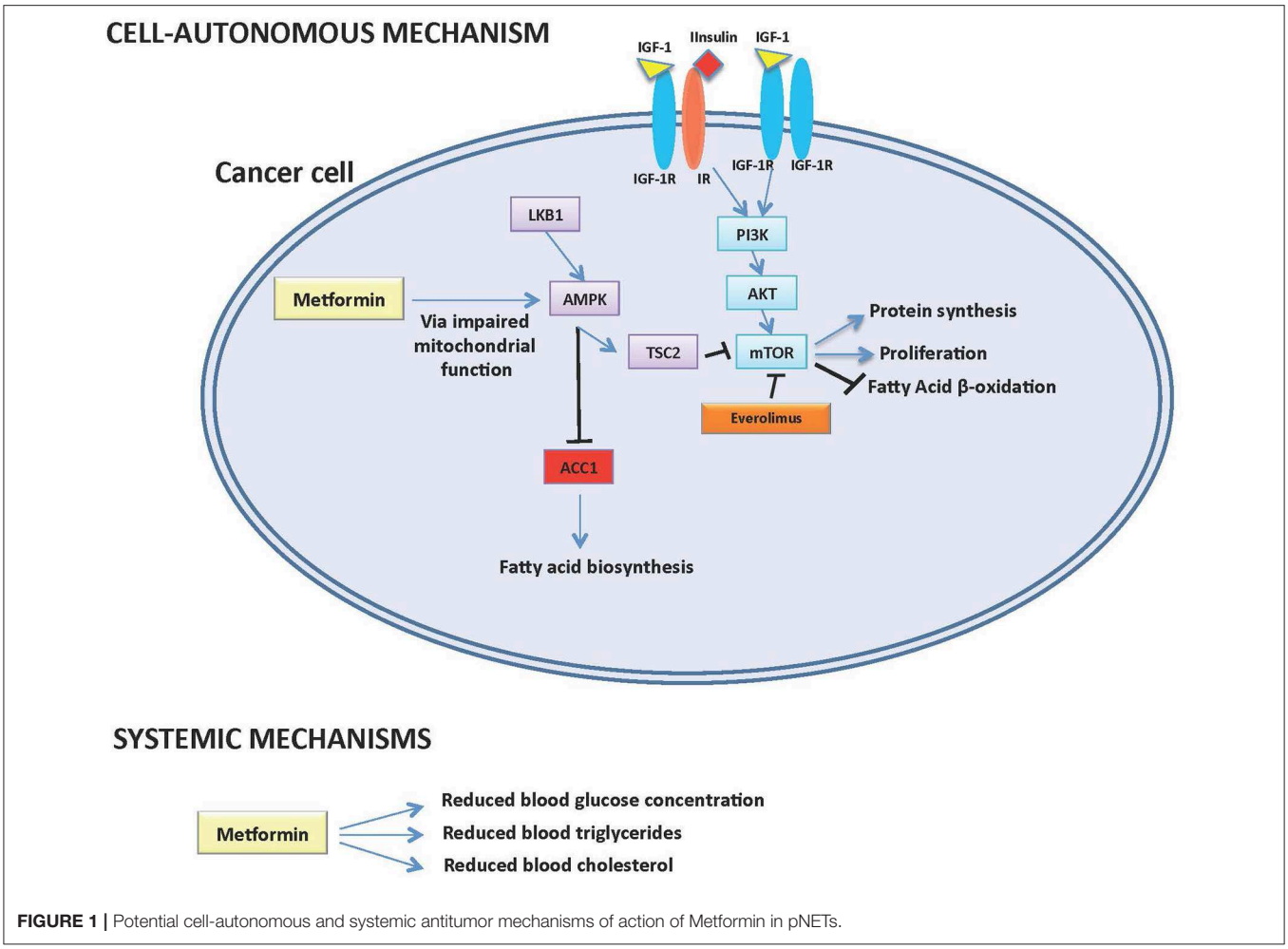

FIGURE 1 | Potential cell-autonomous and systemic antitumor mechanisms of action of Metformin in pNETs.

Here we review and discuss preclinical and clinical studies supporting a potential role of metformin in the treatment of pNETs. We also discuss how ongoing trials could elucidate a potential role of metformin in combination with established anti-pNET therapies.

METHODS

We searched in PubMed database using the following string: "(biguanides OR metformin) AND (pancreatic neuroendocrine tumors OR pNETs OR neuroendocrine tumors.)" Our search strategy produced a total number of 82 articles. Among them, we selected for this review article those original preclinical and clinical papers that investigated the metformin in preclinical pNET models or in patients with advanced pNETs.

Evidence of Metformin Activity Against pNETs

Preclinical Evidence

Metformin has demonstrated anti-pNET activity in preclinical studies (8–10). For instance, metformin impaired cell migration capacity and reduced the survival of BON-1 (serotonin-secreting) and QGP-1 (non-hormone-secreting) pNET cell lines (8). In BON-1 but not in QGP-1 cells, metformin strongly inhibited the transcription of insulin receptor gene (*INSR*), and also reduced levels of phosphorylated ERK and AKT (8). In another study, metformin inhibited the mTORC1/S6K/S6 pathway when used in the 1–10 mM concentration range, and reduced cell viability without inducing apoptosis (9). Due to the central role of the INSR-IGFR1/PI3K/AKT/mTOR pathway in pNET cell growth and proliferation, the ability of metformin to inhibit this axis via AMPK activation may be responsible for its cell-autonomous anticancer effects, as well as for a potentially synergistic antitumor activity between mTORC1 inhibitors and metformin.

Nevertheless, the following issues strongly limit the clinical translatability of *in vitro* studies published so far: (1) metformin concentrations used in cell growth media are in the range of mM (usually 1–20 mM), i.e., by far higher than those that can be reached in patient blood with commonly-used and safe metformin dosages 4–15 μM (11). This limitation highlights the importance of repeating crucial experiments with more physiological metformin concentrations; (2) the contribution of metformin-induced

systemic metabolism modifications on its anticancer activity cannot be assessed in *in vitro* studies. Since metformin may inhibit cancer growth by modifying systemic metabolism, and in particular by lowering the blood concentration of glucose, insulin, and lipids, this limitation is especially important in the perspective of their clinical translation.

Clinical Data

The first indication of a potential impact of metformin on the outcome of patients with advanced pNET patients came from a small retrospective study that we conducted in 31 patients. In this study, we found that metformin use in diabetic patients was associated with significantly longer PFS when compared to diabetic patients not receiving metformin, or to non-diabetic patients (**Table 1**)(12).

Aiming to expand these preliminary data, we conducted a large retrospective, multicentric study involving 24 Italian centers and 445 patients (13). In this study, we found that diabetic patients treated with metformin had remarkably longer PFS (44.2 months) when compared to other diabetics (20.8 months) or to non-diabetic patients (15.1 months) (**Table 1**). Importantly, the positive impact of metformin was observed regardless of the concomitant anticancer treatment (SA or everolimus plus SA), and was independent from other known prognostic variables, including the presence of liver metastases or having undergone previous surgery of the primary tumor. In our study, patient glycemic status was not independently associated with PFS, thus suggesting that plasma glucose levels do not affect treatment efficacy (13). We also provided indirect arguments supporting the conclusion that blood insulin concentration is unlikely to have an effect on patient outcomes. Therefore, we finally hypothesized that the anticancer role of metformin in advanced pNETs is more likely to be mediated by cell-autonomous antitumor effects.

On the other hand, we recently published results of a study indicating that modifications of lipid metabolism could be implicated in metformin anticancer properties. Indeed, in a 58 patients with advanced pNETs treated with everolimus, we found that the precocious (within 3 months from treatment initiation) onset of hypertriglyceridemia, or increased cholesterol levels during the whole treatment course, are associated with significantly lower PFS independently from metformin use (**Table 1**) (14). We also found that high intratumor levels of Acetyl-CoA Carboxylase 1 (ACC1) enzyme, the limiting-step enzyme in the fatty acid *de novo* biosynthesis pathway, correlate with lower everolimus efficacy [**Figure 1**; (14)]. Since metformin not only affects systemic glucose metabolism, but it is also capable of lowering plasma triglycerides (15) and/or of causing inhibition of ACC1 in AMPK-mediated manner in cancer cells (16), the observed association of metformin use and significantly longer patient PFS could be mediated by metformin effects on systemic/tumor lipid metabolism (**Figure 1**). Prospective studies are needed to test this hypothesis, as well as to distinguish between an indirect (i.e., mediated by modifications of systemic metabolism) and a direct, cell-autonomous anticancer effect of metformin in pNETs.

Ongoing Studies

While retrospective analyses clearly indicate that metformin use in diabetic patients with advanced pNETs is associated with better clinical outcomes, no prospective studies have investigated metformin activity/efficacy in combination with standard antitumor treatments so far. Moreover, it is currently unclear if also pNET patients who are not diabetics ma benefit from metformin treatment.

T2DM is characterized by the concomitancy of hyperglycemia, insulin resistance, and hyperinsulinemia (17). Furthermore, T2DM is frequently associated with metabolic syndrome, which is defined by the presence of glucose intolerance, hypertriglyceridemia, low HDL cholesterol levels, obesity, and high blood pressure (18). Therefore, the presence of both glucose and lipid metabolism dysregulation is common in T2DM patients (17). Metformin is effective in reducing hyperglycemia and insulin resistance occurring in T2DM patients, and also reduced blood triglyceride and cholesterol concentration in some studies (15).

At our Institution, the single-arm, open label MetNet1 trial (NCT02294006) is currently enrolling patients with advanced pNETs regardless of their diabetic status (19). Patients enrolled in this trial are prescribed upfront treatment with SAs plus everolimus plus metformin, up to a maximum daily dosage of 2,000 mg. The primary objective of the study is to evaluate the efficacy of the experimental treatment, as defined as median PFS. Other study objectives consist in testing the tolerability of the experimental treatment, as well as its effects on systemic metabolism. Of note, metformin does not significantly alter glucose and lipid metabolism in patients with normal baseline profiles. Therefore, if metformin anticancer effects in pNET patients are mainly mediated through modifications of systemic metabolism, diabetic pNETs patients, who more frequently have deregulated glucose and lipid metabolism, may benefit from metformin significantly more than non-diabetic ones. Conversely, if metformin mainly acts through a cell-autonomous anticancer effect, diabetic, and non-diabetic patients should benefit from metformin treatment in a similar way. Discarding between these two possibilities will be crucial to properly select pNET patients who are the best candidates to receive metformin in combination with standard anticancer treatments.

DISCUSSION

Based on the available preclinical and retrospective clinical evidence, metformin administration promises to provide clinical advantage when used in combination with established anticancer treatments, such as SAs and everolimus, in patients with advanced pNETs (12–14). Since plasma glucose levels have not been found to be associated with pNET patient prognosis, it is unlikely that the major effect of metformin is mediated by its ability to reduce patient glycemia. On the other hand, emerging data suggest that the effect of metformin could be mediated through its impact on systemic lipid metabolism, especially in patients treated with mTOR inhibitors, which increase

TABLE 1 | Published and ongoing studies of metformin in pNET patients.

References	Study design	N. patients	Status	Study findings
Pusceddu et al. (12)	Retrospective	31	Completed	Diabetic pNET patients taking metformin have significantly longer PFS when compared with non-diabetics or diabetics pNET patients treated with other antidiabetic therapies
Pusceddu et al. (13)	Retrospective	445	Completed	Diabetic pNET patients taking metformin have significantly longer PFS (44.2 months) when compared with non-diabetics (15.1 months) or diabetics pNET patients treated with other antidiabetic therapies (20.8 months). The impact of metformin on patient PFS was independent of other clinically relevant variables
Vernieri et al. (14)	Retrospective	58	Completed	Hypertriglyceridemia and hypercholesterolemia are associated with significantly worse PFS in advanced pNET patients treated with everolimus
Pusceddu et al. (NCT02294006) (14)	Prospective	43	Ongoing	N.A.

PFS: progression-free survival; pNET: pancreatic neuroendocrine tumor.

triglyceride and cholesterol concentration in a significant proportion of patients [**Figure 1**; (14)]. However, these data need to be confirmed in larger retrospective and, in case, in prospective studies. Moreover, a direct, cell-autonomous anticancer effect of metformin against pNETs cannot be excluded, even though metformin concentrations that are active in *in vitro* studies can be hardly reached in patients' blood (**Figure 1**).

Crucial advantages of metformin consist in low drug costs and excellent tolerability at dosages that are commonly used for the treatment of T2DM. However, the tolerability of metformin in combination with standard anticancer treatments needs to be established yet. For instance, the metformin-everolimus combination could increase the risk of everolimus-induced diarrhea. The ongoing NCT02294006 trial will clarify if metformin is a safe and well-tolerated drug when combined with SAs plus everolimus (19). While the risks of specific pharmacological metformin-including combinations cannot be ignored, metformin could also prevent or reduce alterations of glucose and lipid metabolism that are often detected in patients with advanced pNETs, especially those treated with everolimus (4, 14).

While published clinical studies indicate a potentially relevant advantage from adding metformin to standard anti-pNET treatments, prospective studies are necessary before concluding that metformin might provide a true clinical benefit. Indeed, retrospective studies have important limitations that may lead to incorrect conclusions. For instance, metformin use had been associated with longer survival in patients with advanced pancreatic exocrine adenocarcinomas in retrospective studies (20, 21); however, three recent randomized trials showed no benefit from adding metformin to first- or second-line chemotherapy in this patient population (22–24). Different factors may account for discrepancies between retrospective and prospective studies, including the reporting bias, immortal time bias, and the fact that metformin is only taken by patients with T2DM in retrospective studies (13, 25). In the case of pNETs, the impact of the immortal time bias could be especially important: indeed, pNET patients receiving metformin for T2DM treatment could be selected for being exposed to SAs and/or everolimus for longer periods, or for having undergone previous pancreatic surgery, i.e., all clinical characteristics associated with better patient prognosis independently from metformin use.

Another crucial issue in the debate around the use of metformin as an anticancer agent consists in clarifying its potential antitumor activity in patients who are not diabetics. Since in our retrospective study the diabetic status was not associated with patient PFS independently from other prognostic factors, it is reasonable to hypothesize that metformin could improve patient prognosis independently from its impact on glucose metabolism but, more reasonably, through its effects on other metabolic pathways or through cell-autonomous anticancer effects. In both cases, we would expect similar anticancer activity from metformin in patients with and without diabetes. Prospective studies including both diabetic and non-diabetic patients, as well as correlative analyses between kinetics of blood triglyceride/cholesterol concentration and treatment efficacy, will be crucial to clarify the role of metformin-induced metabolic modifications on its anticancer activity. On the other hand, preoperative, window-of-opportunity trials with single-agent metformin in patients candidate to surgery could represent the ideal context to explore potential metformin cell-autonomous antitumor properties, as well as to clarify if commonly used dosages of this compound are sufficient to reach therapeutic intratumor concentrations.

To date, the strongest rationale exists for combining metformin with everolimus, which could synergize at a molecular (i.e., by strengthening inhibition of the PI3K/AKT/mTOR pathway and inhibiting cancer cell anabolism) and systemic (i.e., by reducing blood glucose, triglyceride, and cholesterol concentration) levels. However, future preclinical and, in case, clinical studies should investigate metformin in combination with other therapies that are standard-of-care in pNET patients, such as SSAs and PPRT.

AUTHOR CONTRIBUTIONS

All authors (CV, FB, and SP) have contributed to conception or design of the paper, as well as to the writing of the manuscript and its critical revision.

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Pretreatment Inflammatory Indexes as Prognostic Predictors of Survival in Patients Suffering From Synovial Sarcoma

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Background: Inflammatory indexes have been considered as important prognostic factors in various types of cancers. This study aimed to evaluate prognostic values of neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR) in patients with synovial sarcoma (SS).

Methods: One hundred and three patients diagnosed with SS were collected during 2006–2017 and divided into high or low NLR, PLR, and LMR groups based on receiver operating characteristic curve analysis. Data of clinical variables were collected for univariate and multivariate analyses. The Kaplan–Meier method was used to analyze OS and PFS of SS patients and significance was evaluated by the log-rank test.

Results: The optimal cut-off values of NLR, PLR, and LMR were 2.70, 154.99, and 4.16, respectively. Univariate analyses identified resection surgery, distant metastasis, NLR, PLR, and LMR as the potential predictors of progression-free survival (PFS) and overall survival (OS). In the multivariate analyses, NLR was independent predictors for OS (HR 5.074, 95% CI 1.200–21.463, $p = 0.027$). Resection surgery, metastasis and LMR was independent predictors for PFS (HR 5.328, $p = 0.017$; HR 3.114, $p = 0.04$ and HR 0.202, $p = 0.025$, respectively).

Conclusion: Resection surgery, distant metastasis, NLR, and LMR were independent prognostic factors of PFS and OS in patients with synovial sarcoma. Surgery as an effective treatment strategy, other than radiotherapy and chemotherapy, can significantly prolong survival of synovial patients. Clinical utility of these inflammatory biomarkers should be validated in a larger sample size study.

Keywords: synovial sarcoma, inflammatory biomarkers, neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), survival

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INTRODUCTION

Soft tissue sarcomas (STSs) are mesenchymal malignant tumors, accounting for <1% of all malignant tumors and 2% of all cancer-related deaths (1). Although synovial sarcoma (SS) accounts for only ∼5 to 10% of all STSs, it is the commonest non-rhabdomyosarcomatous soft tissue sarcoma in adolescent and young adults (1, 2). SS was once thought to originate from synovial cells due to its

frequent occurrence in soft tissue around joint. However, it has been found in almost every part of the body with a rare frequency and the specific cellular origin remains unclear (3, 4). SS is generally considered as a high-grade sarcoma, with 5-, 10-, and 15-year survival rates survival rates of ∼60, 50, and 45%, respectively (5). SS tends to occur in young people, with a slight male predominance, and mostly affects extremities (>80%) (6). The tumors can be divided into three histological types: biphasic (consist of both spindle and epithelioid cells), monophasic (only spindle cells component) and poorly differentiated (containing small round cells). Despite the morphological difference, they are histogenetically similar through the presence of the t(X;18)(p11.2;q11.2) translocation (7, 8).

It is known that tumor size (<5 cm), age of patients (<20 years old), radiotherapy and complete resection surgery are important positive prognostic factors for patients with SS (5, 9). Whereas, smaller SSs unexpectedly have a poor prognosis during occasional cases (9). In another cohort, age <35 years is a main predictor for patients' prognosis (6). Treatment strategies for SS involve surgery, chemotherapy and radiotherapy. Surgery is an optimal choice for localized tumor, which is usually combined with radiotherapy. Radiotherapy aims to decrease tumor size and help in delaying local invasion. Patients with standard care of surgery and radiotherapy usually have a good chance to control localized disease (10). Although SS is considered to be sensitive to chemotherapy, especially to alkylating agents like ifosfamide and doxorubicin, when compared with other adult soft tissue sarcoma, the response rate still remains about 50% (11, 12). Whereas, routine administration of chemotherapy is of no benefit in reducing systemic relapse in pediatric patients (10). The therapeutic effects of both chemotherapy and radiotherapy vary from different types and stages of SS (13). Therefore, identifying high-risk SS through a different way might be helpful in management of this disease.

Recently, increasing evidence has revealed that systemic inflammatory response plays a remarkable role in prognosis of various malignant tumors, including colorectal cancer, breast cancer, gastric cancer, esophageal cancer, ovarian cancer and pancreatic cancer (14–19). For soft tissue sarcoma, previous investigates have also indicated that inflammatory indexes, such as neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR) and absolute lymphocyte count (ALC), are independent prognostic biomarkers for osteosarcoma, Ewing sarcoma and rhabdomyosarcoma (20–22). Recent years, several studies have focused on prognostic factors for synovial sarcoma. High NLR was found to be a reliable prognostic factor which was associated with worse survival for synovial sarcoma patients (23, 24).

The aim of this study was to estimate the prognostic values of not only pre-treatment NLR, but also PLR and LMR in SS patients and identify high-risk patients for better management.

PATIENTS AND METHODS

Patients

The Medical Ethics Committee of West China Hospital approved this study before this study launch. We retrospectively reviewed the medical records of all newly diagnosed synovial sarcoma patients between January, 2005 and December, 2017 in West China Hospital. The inclusion criteria were as follows: (a) patient with SS confirmed by histopathology; (b) patients without previous anti-cancer treatment, including surgery resection, chemotherapy and radiotherapy; (c) patients with informed consent. The exclusion criteria included: (a) patients with obvious infection or autoimmune diseases; (b) patients with hematologic diseases; (d) patients suffered from other malignant diseases; (e) patients without sufficient data for further analysis. Finally, 103 patients were included in this study. Each patient was followed up regularly until death or December 2017. The follow-up interval varied from 6 month to 1 year.

Data Extraction and Inflammatory Indexes Analysis

Clinical features, including age, sex, tumor location, metastasis at diagnosis, tumor size, treatment strategy, and laboratory index values, such as neutrophil counts, lymphocyte counts, platelet counts, monocyte counts, LDH, were extracted from the medical records of the enrolled patients. OS was measured as the period between the date of diagnosis of SS and the date of death. PFS was calculated from the date of diagnosis to the date of disease relapse and progression. The date of last follow-up was used for drop-out patients. NLR and PLR were defined as the ratio of absolute neutrophil counts and platelet counts divided by the absolute lymphocyte counts, respectively. LMR was defined as the absolute lymphocyte counts divided by the absolute monocyte counts. Patients with complete resection surgery were refer to those who have undertaken surgery treatment, whereas patients with margin status R1 or R2 were refer to no surgery treatment.

Statistical Analysis

Receiver operating characteristic (ROC) curve was applied to evaluate the sensitivity of the inflammatory indexes and Youden index was identified as the optimal cut-off value. Student's *t*-test was used to exam the difference of continuous variables. Comparison of categorical variables, Chi-square test or the Fisher exact test was applied. Survival curves were plotted by Kaplan–Meier analysis and Log-rank test was performed to identify the significance of the difference. Significant variables for OS or PFS were identified by univariate analysis and then further evaluated by multivariate analysis using Cox's proportional hazard regression analysis. *P*-values were based on two-tail test and <0.05 were considered statistically significant. All statistical analysis was performed by using SPSS version 19.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Baseline Characteristics

A total of 149 patients with synovial sarcoma were identified from our database and 103 patients were finally enrolled. The cut-off values of NLR, PLR, and LMR were 2.70, 154.99, and 4.16, respectively. The baseline characteristics of patients are shown in **Table 1**.

SS tended to occur in younger people, with median age of patients was 37 (range 1–78) years. There were 54 (52.4%) males

and 49 (47.6%) females. Most patients (95, 92.2%) received surgery, whereas only 19 (18.4%) and 32 (31.1%) received radiotherapy and chemotherapy, respectively. During the follow-up period, 41 (39.8%) patients experienced disease relapse and 22 (21.4%) patients had distant metastasis. Of note, metastasis was significantly associated with NLR, PLR and LMR. Patients with high NLR, PLR and lower LMR were likely to develop distant metastasis. Pathological results suggested extremities were the most common sites for SS (56, 54.4%). Eighteen (17.5%) and 29 (28.2%) patients had tumor located in internal organ and trunk, respectively. Of the entire patients, 64 (62.1%) patients had tumors larger than 5 cm.

The median overall survival (OS) and median progression-free survival (PFS) was 44.0 months (95% confidence interval [CI] 37.0–57.0) and 25.0 months (95% CI 14.6–36.0), respectively. We explored associations of NLR, PLR, and LMR with these baseline characteristics and results suggested chemotherapy, distant metastasis and tumor location were statistically significantly associated with NLR, PLR, or LMR ($p < 0.05$).

Univariate Analyses and Multivariate Analyses

We investigated the associations between patients' baseline characteristics, including NLR, PLR, and LMR, and survival by using Cox's proportional hazard regression analysis. Univariate analyses indicated that resection surgery, distant metastasis, tumor location, NLR, PLR, and LMR were closely correlated with prognosis of patients ($p < 0.1$). Furthermore, multivariate analyses of OS and PFS were performed including markers mentioned above to identify independent predictor for survival (**Tables 2**, **3**).

TABLE 1 | Baseline characteristics of the patients with synovial sarcoma.

Clinical parameters	Total	NLR		*p*-value	PLR		*p*-value	LMR		*p*-value
	N = 103	<2.70	≥2.70		<154.99	≥154.99		≥4.16	<4.16	
	n (%)	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Median age, years (range)	37 (1–78)	35 (17–78)	37 (1–74)	0.890	37 (1–74)	33 (13–78)	0.280	36 (1–70)	37 (17–78)	0.636
Gender										
Female	49 (47.6)	39 (53.4)	10 (33.3)	0.083	39 (52.0)	10 (35.7)	0.184	37 (54.4)	12 (34.3)	0.063
Male	54 (52.4)	34 (46.6)	20 (66.7)		36 (48.0)	18 (64.3)		31 (45.6)	23 (65.7)	
Surgery										
No	8 (7.8)	5 (6.8)	3 (10.0)	0.689	5 (6.7)	3 (10.7)	0.680	5 (7.4)	3 (8.6)	1.000
Yes	95 (92.2)	68 (93.2)	27 (90.0)		70 (93.3)	25 (89.3)		63 (92.6)	32 (91.4)	
Radiotherapy										
No	84 (81.6)	60 (82.2)	24 (80.0)	0.785	63 (84.0)	21 (75.0)	0.391	57 (83.8)	27 (77.1)	0.430
Yes	19 (18.4)	13 (17.8)	6 (20.0)		12 (16.0)	7 (25.0)		11 (16.2)	8 (22.9)	
Chemotherapy										
No	71 (68.9)	56 (76.7)	15 (50.0)	0.010	57 (76.0)	14 (50.0)	0.016	51 (75.0)	20 (57.1)	0.075
Yes	32 (31.1)	17 (23.3)	15 (50.0)		18 (24.0)	14 (50.0)		17 (25.0)	15 (42.9)	
Relapse										
No	62 (60.2)	44 (60.30)	18 (60.0)	1.000	46 (61.3)	16 (57.1)	0.822	40 (58.8)	22 (62.9)	0.832
Yes	41 (39.8)	29 (39.7)	12 (40.0)		29 (38.7)	12 (42.9)		28 (41.20	13 (37.1)	
Metastasis										
No	81 (78.6)	63 (86.3)	18 (60.0)	0.007	65 (86.7)	16 (57.1)	0.002	58 (85.3)	23 (65.7)	0.040
Yes	22 (21.4)	10 (13.7)	12 (40.0)		10 (13.3)	12 (42.9)		10 (14.7)	12 (34.3)	
Tumor location										
Internal organs	18 (17.5)	10 (13.7)	8 (26.7)	0.062	9 (12.0)	9 (32.1)	0.011	6 (8.8)	12 (34.3)	0.005
Extremities	56 (54.4)	45 (61.6)	11 (36.7)		47 (62.70	9 (32.1)		42 (61.8)	14 (40.0)	
Trunk	29 (28.2)	18 (24.7)	11 (36.7)		19 (25.3)	10 (35.7)		20 (29.4)	9 (25.7)	
T stage[†]										
<5 cm	24 (23.3)	20 (31.3)	4 (16.7)	0.193	20 (30.30	4 (18.2)	0.408	18 (30.5)	6 (20.7)	0.447
≥5 cm	64 (62.1)	44 (68.8)	20 (83.3)		46 (69.7)	18 (81.8)		41 (69.5)	23 (79.3)	
LDH										
<169.5	52 (50.5)	39 (53.4)	13 (43.3)	0.391	42 (56.0)	10 (35.7)	0.079	38 (55.9)	14 (40.0)	0.149
≥169.5	51 (49.5)	34 (46.6)	17 (56.7)		33 (44.0)	18 (64.3)		30 (44.1)	21 (60.0)	

[†] *88 were available. NLR neutrophil-lymphocyte ratio, PLR platelet-lymphocyte ratio, LMR lymphocyte-monocyte ratio, LDH lactate dehydrogenase.*

TABLE 2 | Summary of univariate and multivariate analysis for OS in patients with synovial sarcoma.

Parameter	Average OS	95% CI	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender						
Male	75.6	67.6–83.6	1.000	0.628	–	–
Female	89.4	80.1–98.6	0.782 (0.289–2.118)		–	–
Age						
≥37	86.0	76.0–96.1	1.000	0.542	–	–
<37	82.0	74.4–89.5	0.735 (0.274–1.976)		–	–
Surgery						
No	58.7	28.9–88.6	1.000	0.058	1.000	0.103
Yes	89.6	82.9–96.3	0.295 (0.084–1.041)		3.210 (0.791–13.027)	
Radiotherapy						
No	88.1	80.8–95.3	1.000	0.610	–	–
Yes	84.5	68.8–100.3	1.342 (0.433–4.162)		–	–
Chemotherapy						
No	89.9	82.6–97.3	1.000	0.322	–	–
Yes	82.6	69.7–95.5	1.648 (0.613–4.426)		–	–
Relapse						
No	90.2	82.1–98.4	1.000	0.243	–	–
Yes	83.3	72.0–94.7	1.804 (0.670–4.856)		–	–
Metastasis						
No	93.3	87.0–99.7	1.000	0.009	1.000	0.165
Yes	70.6	53.6–87.6	3.713 (1.392–9.908)		2.331 (0.706–7.699)	
Tumor location						
Internal organs	58.5	41.5–75.4	1.000	0.062	1.000	
Extremities	89.9	81.6–98.2	0.326 (0.109–0.977)	0.045	0.391 (0.097–1.582)	0.188
Trunk	93.2	82.9–103.6	0.241 (0.059–0.980)	0.047	0.258 (0.056–1.183)	0.081
T stage						
<5 cm	85.8	77.5–94.1	1.000	0.232	–	–
≥5 cm	77.3	69.4–85.3	2.493 (0.557–11.152)		–	–
LDH						
<169.5	88.4	79.2–97.7	1.000	0.924	–	–
≥169.5	79.0	70.8–87.2	1.049 (0.393–2.798)		–	–
NLR						
<2.70	86.0	80.7–91.3	1.000	0.003	1.000	**0.027**
≥2.70	71.0	55.1–86.9	4.651 (1.688–12.811)		5.074 (1.200–21.463)	
PLR						
<154.99	84.0	78.1–90.0	1.000	0.037	1.000	0.167
≥154.99	76.0	60.4–91.5	2.832 (1.062–7.553)		3.195 (0.615–16.589)	
LMR						
<4.16	71.2	56.5–85.9	1.000	0.002	1.000	0.056
≥4.16	96.1	90.3–101.9	0.190 (0.066–0.547)		0.280 (0.076–1.035)	

OS overall survival, LDH lactate dehydrogenase, NLR neutrophil-lymphocyte ratio, PLR platelet-lymphocyte ratio, LMR lymphocyte-monocyte ratio.

Treatment strategies for SS remained unclear, however, our results suggested patients received resection surgery had better PFS (87.2 vs. 44.5 months, $p = 0.056$) and OS (89.6 vs. 58.7 months, $p = 0.058$) than those without resection surgery. Resection surgery was shown to be an independent indicator for PFS (hazard ratio [HR] 5.328, 95% CI 1.349–21.041, $p = 0.017$), not for OS. Patients without distant metastasis shared favorable PFS (81.5 months vs. 63.5 months, $p < 0.01$ and OS (93.3 months vs. 70.6 months, $p < 0.01$). Metastasis was independent indicator for PFS (HR 3.114, 95% CI 1.054–9.199, $p = 0.04$). SS that initially occurred in internal organs, such as lung, kidney and mediastinum revealed poor outcomes, with shorter OS and PFS (compared to extremities, 58.5 vs. 89.9 months, $p = 0.045$ and 38.2 vs. 88.3 months, $p = 0.104$). Compared to patients with higher NLR and PLR, patients in lower NLR and PLR groups were shown to have better PFS (82.7 months vs. 67.0 months, $p <$

TABLE 3 | Summary of univariate and multivariate analysis for PFS in patients with synovial sarcoma.

Parameter	Average PFS	95% CI	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender						
Male	73.9	54.9–82.9	1.000	0.809	–	–
Female	85.3	73.6–97.0	1.130 (0.420–3.038)		–	–
Age						
≥37	82.8	71.4–94.3	1.000	0.619	–	–
<37	74.0	65.8–82.3	0.778 (0.290–2.090)		–	–
Surgery						
No	P	20.8–68.2	1.000	0.056	1.000	**0.017**
Yes	87.2	79.7–94.7	0.292 (0.083–1.031)		5.328 (1.349–21.041)	
Radiotherapy						
No	86.8	78.9–94.8	1.000	0.643	–	–
Yes	73.3	58.0–88.7	1.309 (0.420–4.074)		–	–
Chemotherapy						
No	88.2	79.7–96.6	1.000	0.269	–	–
Yes	69.5	56.7–82.2	1.748 (0.650–4.698)		–	–
Relapse						
No	90.5	82.4–98.5	1.000	0.074	1.000	**0.034**
Yes	48.1	35.8–60.4	2.511 (0.915–6.892)		3.301 (1.094–9.964)	
Metastasis						
No	81.5	75.2–87.8	1.000	0.004	1.000	**0.040**
Yes	63.5	42.5–84.6	4.186 (1.568–11.179)		3.114 (1.054–9.199)	
Tumor location						
Internal organs	38.2	24.5–52.0	1.000	0.227	–	–
Extremities	88.3	78.8–97.8	0.360 (0.105–1.235)	0.104	–	–
Trunk	76.3	66.0–86.6	0.384 (0.103–1.430)	0.154	–	–
T stage						
<5 cm	82.7	71.7–93.7	1.000	0.240	–	–
≥5 cm	71.6	62.8–80.5	2.456 (0.549–10.983)		–	–
LDH						
<169.5	85.7	75.1–96.0	1.000	0.870	–	–
≥169.5	76.2	66.7–85.6	1.085 (0.407–2.893)		–	–
NLR						
<2.70	82.7	76.3–89.1	1.000	0.003	1.000	0.098
≥2.70	67.0	49.3–84.6	4.653 (1.686–12.847)		3.361 (0.801–14.102)	
PLR						
<154.99	80.4	73.5–87.4	1.000	0.027	1.000	0.226
≥154.99	72.2	54.7–89.6	3.040 (1.137–8.125)		2.671 (0.544–13.116)	
LMR						
<4.16	69.0	52.8–85.1	1.000	0.003	1.000	**0.025**
≥4.16	83.7	77.5–89.8	0.199 (0.069–0.574)		0.202 (0.050–0.821)	

PFS progression-free survival, LDH lactate dehydrogenase, NLR neutrophil-lymphocyte ratio, PLR platelet-lymphocyte ratio, LMR lymphocyte-monocyte ratio.

0.01 and 80.4 months vs. 72.2 months, $p < 0.05$, respectively) and OS (86.0 months vs. 71.0 months, $p < 0.01$ and 84.0 months vs. 76.0 months, $p < 0.05$, respectively). On the contrary lower LMR was a marker for shorter PFS (69.0 vs. 83.7 months, $p < 0.01$) and OS (71.2 vs. 96.1 months, $p < 0.01$). NLR was an independent predictor for OS, with higher NLR associated with poor prognosis (HR 5.074, 95% CI 1.200–21.463, $p = 0.027$). Higher LMR, as an independent indicator for PFS, was significantly associated with better PFS (HR 0.202, 95% CI 0.050–0.821, $p = 0.025$). However, PLR was not independent indicator for either OS or PFS. Other characteristics, including gender, age, radiotherapy, chemotherapy, tumor size and LDH, were not shown to be associated with PFS and OS.

Kaplan-Meier Survival Analysis

Kaplan–Meier curve showed that distant metastasis, NLR, PLR, LMR, and surgery were significantly associated with PFS and OS (**Figures 1–5**).

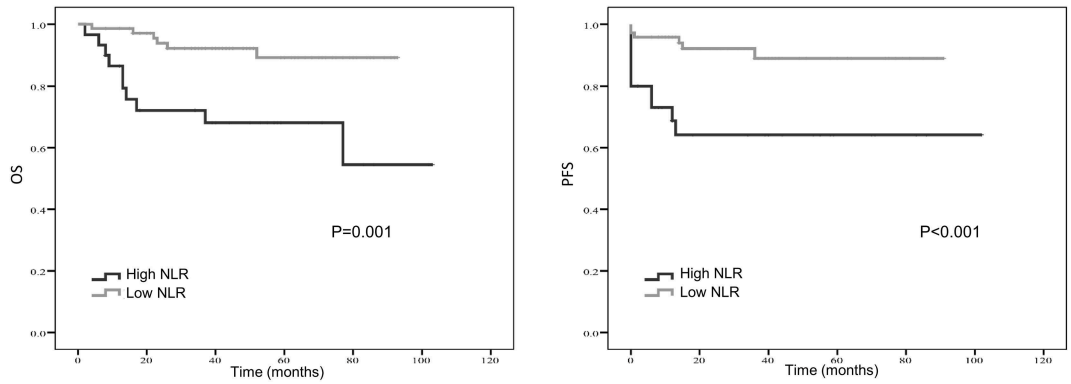

FIGURE 1 | Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) probability according to pre-treatment neutrophil-to-lymphocyte ratio (NLR) level.

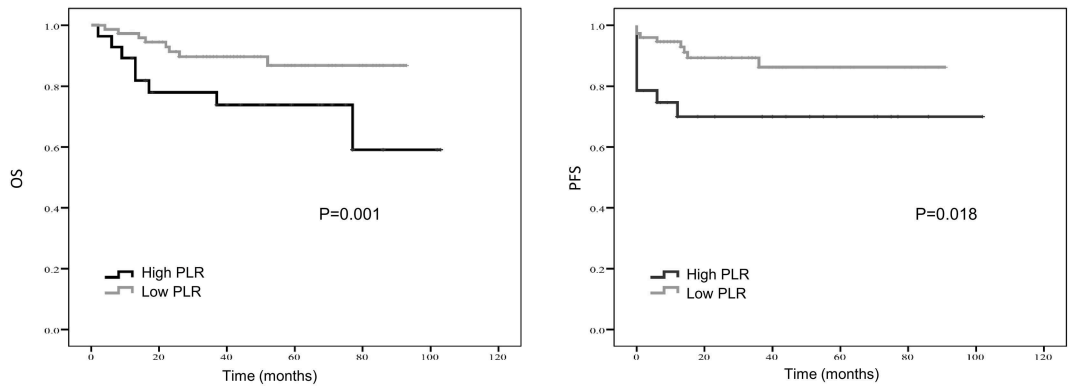

FIGURE 2 | Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) probability according to pre-treatment platelet-to- lymphocyte ratio (PLR) level.

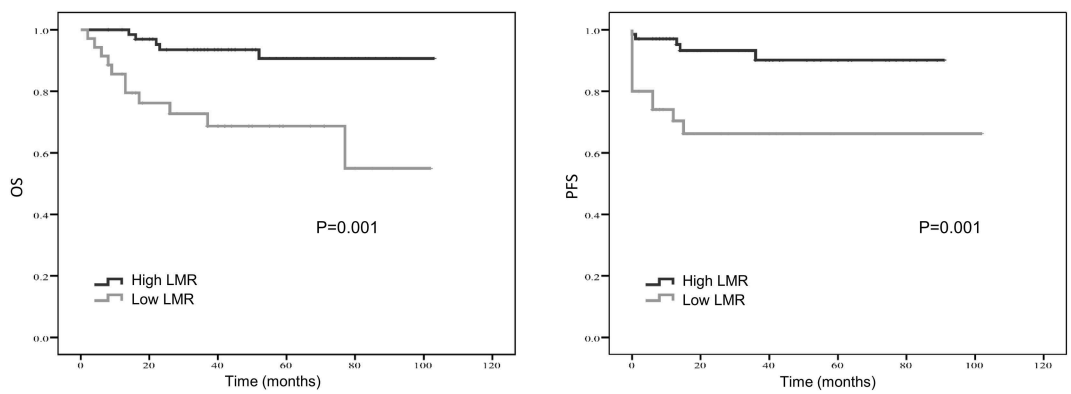

FIGURE 3 | Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) probability according to pre-treatment lymphocyte-to-monocyte-ratio (LMR) level.

DISCUSSION

Inflammatory indexes as prognostic factors for STSs have recently received more and more attention. Not only individual inflammatory markers, such as CRP, and lymphocytes, but also combination of them, such as NLR, PLR and LMR, have been investigated in STSs (20, 22, 25, 26). NLR was found to be a prognostic inflammatory index for synovial sarcoma (SS) (23, 24). In addition, the overall survival of SS still remains unsatisfying (27). Therefore, the aim of

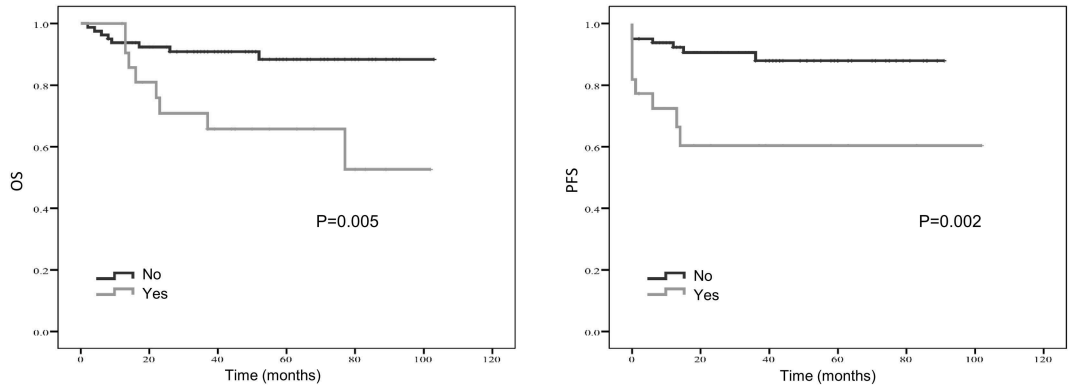

FIGURE 4 | Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) probability according to localized vs. metastatic disease.

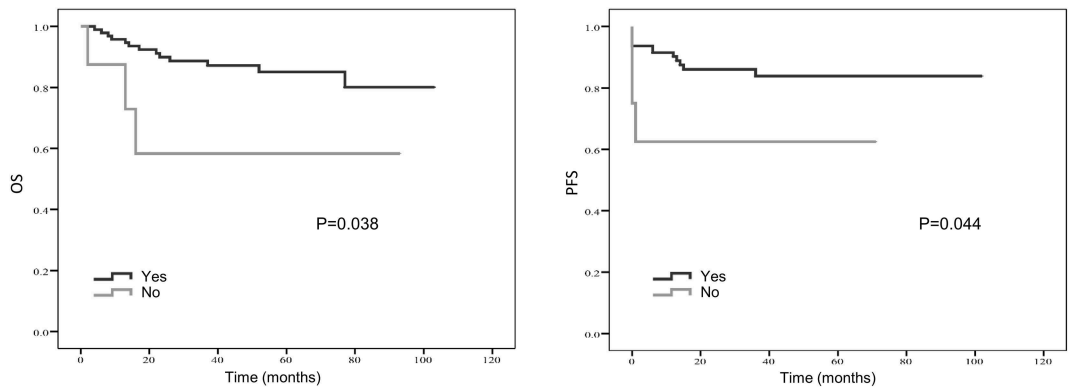

FIGURE 5 | Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) probability according to whether surgery was applied.

this study was to identify more valuable prognostic indexes for SS and to select patients who were at high risk and required more aggressive treatment strategies. Our results suggested that not undergoing complete resection surgery, distant metastasis, high NLR group, high PLR group, and low LMR group were significantly associated with poor prognosis. Whereas, gender, age, radiotherapy, chemotherapy, tumor size, and LDH were not significantly associated with patients' OS and PFS. Our data failed to demonstrate the prognostic values of tumor size, radiotherapy and chemotherapy. This finds were not only limited by small sample size and non-randomized cohorts, but also because of raising debates of administration of chemotherapy (28–30). Previous studies have proved that high NLR, PLR and low LMR are associated poor prognosis in various malignancies (31–35). Current research also confirmed the prognostic values of these three inflammatory indexes in SS.

NLR, PLR, and LMR are derived from the absolute counts of neutrophils, lymphocytes and monocytes, therefore, the ratios of these three groups of cells in tumor microenvironment play a vital role in predicting the prognosis of patients. Myeloid-derived cells, such as neutrophils and monocytes, are the most abundant hematopoietic cells in human body but usually regarded as potent immune suppressors in tumor microenvironment (36). Myeloid-derived suppressor cells (MDSCs) have recently been widely investigated. This group of cells produces a proinflammatory response and promotes angiogenesis and metastasis of tumor (37–39). Derived from circulating monocytes, tumor-associated macrophages have also been proved to be related to tumor cells proliferation, invasion and metastasis (40, 41). In contrast, tumor-infiltrating lymphocytes are considered important in anti-cancer immune response via producing cytokines and inducing cytotoxic cell death (42). Therefore, lymphocytes are thought to be a positive predictor (43, 44). Individual absolute counts of neutrophils and monocytes are suggested to be independent prognosis factors in various cancers (45–47). Meanwhile, it is known that the immune suppressive effect of MDSCs is mainly based on suppressing the activity of T lymphocytes (36). Productions released by MDSCs, such as Arg1 and iNOS, can block T cells and lead to tumor progression and metastasis (48–50). Increased neutrophils, monocytes and decreased lymphocytes are associated with immune suppressive status, therefore, high NLR and low LMR are associated with poor survival outcomes.

Platelets also interact with tumor cells and decreased platelets level is associated with decreased tumor metastasis (51). Tumor cells can gather platelets and protect themselves from cytolysis of NK cells in human blood. This process promotes migration of tumor cells and tumor metastasis (52). Meanwhile, platelets provide a procoagulant surface to help cancer cells escape from immune response, thus promote cancer growth and dissemination (53). Platelets can also activate several signaling pathways within cancer cells, resulting in transition toward a more invasive mesenchymal-like phenotype (54). In accordance with the critical role of lymphocytes in the suppression of tumor progression, high PLR suggests a rather poor prognosis for cancer patients. Although PLR is significantly associated with poor prognosis in univariate analysis, it was not an independent prognostic factor for OS or PFS, which was consistent with previous studies on soft tissue sarcomas and other malignancies (22, 55). One possible reason is that the immune-suppression and tumor-promotion effect of MDSCs are more sustainable and potent than platelets (56), which makes platelet plays a rather small role.

This study investigated the impact of inflammatory indexes on synovial sarcoma and provided an alternative predictive model for prognosis of SS. However, this research still has several limitations. Firstly, due to the rarity of SS and single-center study, the number of patients ($n = 103$) is limited, which may cause selection bias. The subgroup analyses are also limited by the small sample size. Whereas, SS may behavior differently according to age of presence or histological subtypes. Inflammatory indexes are likely to play different roles in the subgroup analyses. Secondly, we just collected relatively a few clinical predictors and some important indicators may be ignored. Therefore, a larger sample size study with more clinical indicators is required to validate our findings. For more precise evaluation, randomized clinical trials are required.

CONCLUSION

In summary, high NLR, high PLR, low LMR, metastasis at diagnosis and no surgery were remarkable risk factors for SS patients. Furthermore, NLR, LMR and metastasis were independent factors for OS and PFS, except for PLR. As a result, NLR and LMR, as inflammatory indexes, were superior to PLR. Surgery could significantly prolong PFS of SS patients. These prognostic indexes might be helpful in making treatment decisions for SS patients with different risks.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

Approval for this retrospective research was obtained from the institutional review board of West China hospital, Sichuan University. Written informed consent was obtained from all included patients.

AUTHOR CONTRIBUTIONS

XM: study concept and design. YC, FM, and QL: acquisition of data. YC and XM: analysis and interpretation of data. YC: drafting of the manuscript. LP and XM: critical revision of the manuscript for important intellectual content. YC, FM, and LP: statistical analysis. XM: study supervision. All authors read and approved the final manuscript.

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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.

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The Efficacy and Safety of Tyrosine Kinase Inhibitors for Von Hippel–Lindau Disease: A Retrospective Study of 32 Patients

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Background: Von Hippel-Lindau (VHL) disease is an autosomal-dominant hereditary cancer syndrome. Currently, studies on tyrosine kinase inhibitor (TKI) therapy for VHL disease are scarce. In this study, we retrospectively evaluated the efficacy and safety of four TKIs in patients with VHL disease.

Methods: Patients diagnosed with VHL disease who were receiving TKIs were recruited. Patients were treated with sunitinib ($n = 12$), sorafenib ($n = 11$), axitinib ($n = 6$), or pazopanib ($n = 3$). The therapeutic response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1.

Results: From July 2009 to September 2018, 32 patients with VHL disease were eligible and included in this study. The median duration of TKI therapy was 22 months (IQR 8.5–44.75), and the median follow-up period was 31.5 months (IQR 13.5–63.5). According to the RECIST, 9 (28%) of 32 patients showed a partial response, 15 (47%) achieved stable disease, and eight exhibited continued disease progression. A partial response was observed in 11 (31%) of 36 renal cell carcinomas, 4 (27%) of 15 pancreatic lesions, and 1 (20%) of five central nervous system (CNS) hemangioblastomas. The average tumor size decreased significantly for renal cell carcinomas ($P = 0.0001$), renal cysts ($P = 0.027$), and pancreatic lesions ($P = 0.003$) after TKI therapy. Common side effects included hand–foot skin reactions, diarrhea, alopecia, thrombocytopenia, and fatigue.

Conclusions: Partial alleviation of VHL disease-related tumors can be achieved by TKI therapies in some patients, providing an alternative treatment strategy, and the side effects of TKIs are acceptable. Larger prospective studies are warranted to further evaluate the efficacy and safety of TKIs in patients with VHL disease.

Keywords: von Hippel-Lindau disease, tyrosine kinase inhibitors, renal cell carcinoma, efficacy, safety, sunitinib, sorafenib, axitinib

INTRODUCTION

Von Hippel-Lindau (VHL) disease (OMIM 193300) is an autosomal-dominant, multiorgan, familial neoplastic syndrome that results from a germline mutation in the *VHL* tumor suppressor gene (1–3). The incidence of the *VHL* mutation is ∼1 in 36,000 live births, and the penetrance is >90% by 65 years of age (3–6). Clinically, VHL disease is characterized by various types of tumors, including central nervous system (CNS) hemangioblastoma (CHB), retinal angioma (RA), renal cell carcinoma (RCC), pancreatic cystic lesions, pancreatic neuroendocrine tumors (PNETs), pheochromocytoma, endolymphatic sac tumors (ELSTs), and epididymal and broad ligament cystadenoma (3, 6, 7). Previously, the prognosis of VHL disease was discouraging, and the median lifespan of patients was reported to be 49 years (8). The most common causes of death were associated with RCCs and CNS hemangioblastomas (8, 9). However, recent studies have reported that the life expectancy of patients with VHL disease has been extended to 64 years (9–11). This improved prognosis may be attributed to several efforts, including earlier diagnosis, active surveillance, and improved treatment of these patients.

In VHL disease, *VHL* mutations lead to the accumulation of hypoxia-inducible factors (HIFs), which activate multiple downstream genes, such as vascular endothelial growth factor (VEGF), erythropoietin, platelet-derived growth factor β (PDGF-β), and transforming growth factor α (TGF-α) (12, 13). Currently, small-molecule tyrosine kinase inhibitors (TKIs), including sunitinib, sorafenib, axitinib, and pazopanib, mainly target the VEGF pathway by inhibiting VEGF ligands or its receptors (14–16). Several studies have reported clinical outcomes in patients with VHL disease treated with TKIs (17–21). A pilot trial by Jonasch et al. (17) assessed the activity and safety of sunitinib in 15 patients with VHL disease, and their results revealed that 6 of the 18 RCCs (vs. none of the CHBs) exhibited a partial response, while the sunitinib dose had to be reduced in 10 patients (17). Only one report has described sorafenib treatment in patients with VHL disease, the results of which showed that low-dose and long-term sorafenib treatment may be an effective option for patients with recurrent RCC (22). Recently, Jonasch et al. completed a prospective study of pazopanib in patients with VHL disease, which revealed that 13 of 31 patients (42%) achieved an objective response and that responses were observed in 31 (52%) of 59 RCCs (20). However, there are currently no studies examining axitinib treatment in patients with VHL disease.

Previous studies have demonstrated that TKIs administered for VHL disease-related tumors may be partially effective and tolerable in most cases. However, the clinical effects of different types of TKIs on various types of tumors in patients with VHL disease are still insufficiently investigated. Thus, in this study, we retrospectively summarized the efficacy and side effects of TKIs for the treatment of patients with VHL disease in a single center. The results showed that TKIs are effective, have acceptable side effects, and are a favorable option for these patients.

MATERIALS AND METHODS

Medical Ethics

This study was approved by the Medical Ethics Committee of Peking University First Hospital (Beijing, China). Informed consent was obtained from patients or their legal guardians.

Patient Recruitment and Assessment

From July 2009 to September 2018, 32 patients with VHL disease (18 males and 14 females) received TKI therapy at Peking University First Hospital. Molecular diagnosis of VHL disease was also conducted in this hospital. The germline *VHL* mutation was identified in 26 of the 32 patients; six patients were diagnosed with VHL disease because the clinical manifestations fulfilled the clinical diagnostic criteria of VHL disease and first-degree relatives carried a germline *VHL* mutation. Therefore, the genotype of *VHL* mutations could be predicted in all patients (**Table 1**). In this retrospective study, patients with VHL disease

TABLE 1 | Demographic characteristics, types of VHL lesions, and VHL mutations in the 32 VHL disease patients treated with TKI therapy.

Characteristic	***n* (%)**
Sex	
Male	18 (56)
Female	14 (44)
Mean age of diagnosis RCC (years)	38.7 ± 10.8 (range 21–59)
Mean age of TKI therapy initiated (years)	41.5 ± 11.2 (range 21–66)
Median duration of TKI therapy (months)	22 (IQR 8.5–44.75)
Median follow-up period (months)	31.5 (IQR 13.5–63.5)
Clinical manifestation	***n* (%)**
Renal cell carcinoma	31 (97)
Renal cyst	22 (69)
Pancreatic tumor or cyst	27 (84)
Pheochromocytoma	6 (19)
Hemangioblastoma	22 (69)
Retinal hemangioma	7 (22)
Endolymphatic sac tumor	1 (3)
Epididymal cystadenoma	4 (21)
VHL mutation	**No. of patients with mutation**
p.R167W	3
p.C162W	2
p.W117G	1
p.N90I	2
p.S88R	1
p.S80I	1
p.S65L	2
p.S65P	1
p.R161*	2
Small indel	8
Large deletion	9

*RCC, renal cell carcinoma; TKI, tyrosine kinase inhibitor; VHL, von Hippel-Lindau; *termination codon.*

were selected non-randomly and mostly included advanced patients (such as those with more metastatic lesions or higher tumor grades), patients who were excluded from partial renal surgery (e.g., bilateral multiple tumors, large tumors, or tumors in proximity to large blood vessels), and patients who had received adjuvant TKI therapy after surgery.

Examination of VHL Mutation

Genomic DNA was extracted from peripheral blood of suspected individuals using QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to instructions. Three coding exons and flanking intronic regions were amplified by polymerase chain reaction using primers as described in our previous publication (23, 24). Direct sequencing was performed to detect missense mutations, splicing mutations, and small indels. Large deletions and duplications were detected by multiplex ligation-dependent probe amplification (MLPA, P016-C2 kit, MRC-Holland, Amsterdam, the Netherlands). All large exon deletions in this study were verified by real-time PCR with primers described by Ebenazer et al. (25).

Drug Dosage

For sunitinib, a dosage of 50 mg/day was given orally for 28 days, followed by a 14-day break per cycle for several cycles. For sorafenib, a dosage of 800 mg/day divided into two doses was administered orally. For axitinib, a dosage of 10 mg/day divided into two doses was administered orally. For pazopanib, a dosage of 800 mg/day was administered orally.

Efficacy and Safety Evaluations

We compared changes in the size of VHL disease-associated tumors before and after TKI therapy in the 32 patients with VHL disease. Baseline and follow-up evaluations of the target lesions were conducted using CT or MRI scans. More than 90% of patients were monitored to assess tumor changes by CT/MRI every 3 months so that complete clinical data could be obtained. The Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) was used to evaluate the therapeutic response. Side effects related to the four TKIs were evaluated using the Common Terminology Criteria for Adverse Events (CTCAE, version 4.0).

Statistical Analysis

Summary statistics, including the mean, SD, IQR, and median, were used to describe patient characteristics. Kaplan–Meier plots and the log-rank test were used for survival analysis. Comparisons of tumor size before and after TKI therapy were performed with paired-sample *t*-tests using SPSS software (version 22.0, IBM-SPSS, Chicago, IL). SAS software (version 9.4) was used to construct the Swimmer plots, which reflected the patients' therapeutic responses to TKI therapy at 3-month intervals. A $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Demographic characteristics, clinical manifestations, and *VHL* mutations of the 32 patients with VHL disease who received TKI therapy are summarized in **Table 1**. The mean age at initiation of TKI therapy was 41.5 ± 11.2 years (range 21–66 years), the median period of TKI therapy was 22 months (IQR 8.5–44.75 months), and the median follow-up period was 31.5 months (IQR 13.5–63.5 months). The most common clinical manifestations of these 32 patients were RCC, pancreatic tumor or cyst, and CNS hemangioblastoma. Truncating mutations were present in 19 patients, and missense mutations were present in 13 patients. In this retrospective study, five patients received more than 5 years of TKI therapy, seven patients received 3–5 years of TKI therapy, 11 patients received 1–3 years of TKI therapy, and nine patients received <1 year of TKI therapy (**Figure 1**).

Overall, after TKI therapy, 9 (28%) of the 32 patients exhibited a partial response, 15 (47%) exhibited stable disease as the best response, and the remaining eight exhibited progressive disease (**Figure 1**). Of the 12 sunitinib-treated patients, 4 (33%) showed a partial response to the therapy, 4 (33%) achieved stable disease, and 4 (33%) exhibited disease progression. Of the 11 sorafenib-treated patients, 3 (27%) showed a partial response to the therapy, 4 (36%) achieved stable disease, and 4 (36%) exhibited disease progression. Of the six axitinib-treated patients, 2 (33%) showed a partial response to the therapy, and the remaining four patients achieved stable disease. All three pazopanib-treated patients achieved stable disease.

The best responses of lesions after TKI therapy as evaluated by the RECIST are shown in **Table 2**. Complete response was not found for any of the lesions. The rate of partial response ranged between 17% (1/6 pheochromocytomas) and 31% (11/36 RCCs). Most lesions were categorized as stable disease; the rate of stable disease ranged between 47% (17/36 RCCs) and 83% (5/6 pheochromocytomas). Progressive disease was not observed in patients with renal cysts, pancreatic tumors or cysts, pheochromocytomas, or CHBs, but it was found in 22% (8/36) of RCCs. The best responses of the different lesions after treatment with the four TKIs are summarized in **Table 2**. Six (40%) of the 15 RCCs presented a partial response, 5 (33%) RCCs were stable, and 4 (27%) RCCs progressed in sunitinib-treated patients; 3 (25%) of the 12 RCCs showed a partial response, 5 RCCs (42%) were stable, and 4 (33%) RCCs progressed in sorafenib-treated patients; 2 (33%) of the six RCCs showed a partial response, and 4 (67%) RCCs were stable in axitinib-treated patients; and all three RCCs were stable in pazopanib-treated patients (**Table 2**). However, the statistical significance of the responses of RCCs to the four TKIs could not be determined due to an insufficient number of cases.

The mean change in the size of the lesions after TKI therapy is summarized in **Table 3**. Specifically, the mean change in size was −19.26, −15.92, −18.46, −28.26, and −18.32% for RCCs, renal cysts, pancreatic tumors or cysts, CHBs, and pheochromocytomas, respectively. The changes were statistically significant, except for the change in CHBs, for which the *P*-value was >0.05. In this study, 12 patients were treated with sunitinib, and 11 patients were treated with sorafenib. Nine patients died during follow-up, mainly because of RCC with lung and/or bone metastasis, and eight patients exhibited disease progression during TKI therapy. The median overall survival duration was 72 months for sunitinib-treated patients and 66 months for sorafenib-treated patients, and the median progression-free

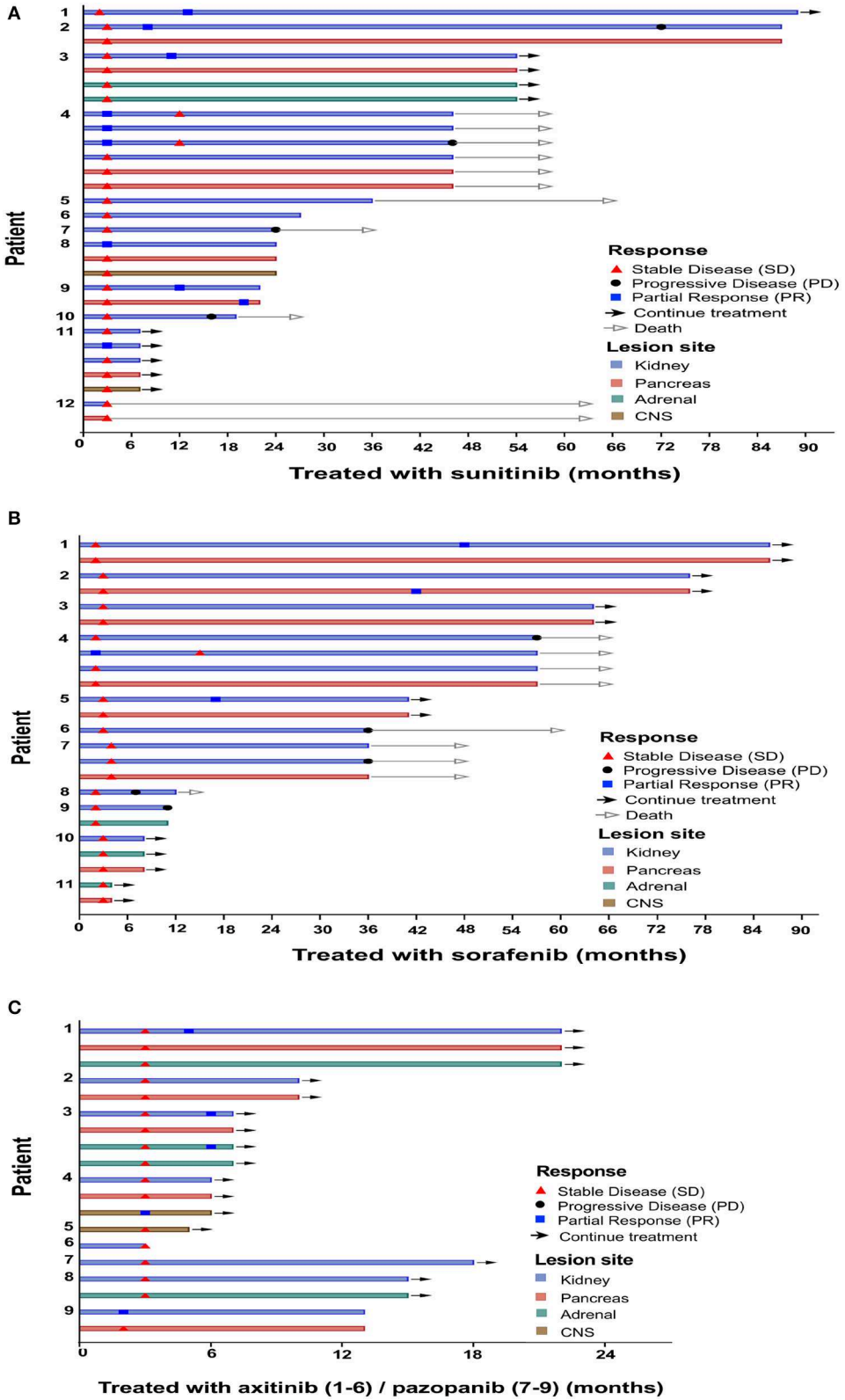

FIGURE 1 | Time point of response appeared. Clinical characteristics per individual. Patients were treated with sunitinib **(A)**, sorafenib **(B)**, axitinib (**C**, patients 1–6), and pazopanib (**C**, patients 7–9).

TABLE 2 | Best response of the lesions by RECIST after treatment of one TKI.

TKI treatment	Clinical characteristic	Evaluable lesions (*n*)	Best response, *n* (%)		
			Partial responsive	Stable	Progressive
Total ($n = 32$)	Renal cell carcinoma	36	11 (31)	17 (47)	8 (22)
	Renal cyst	13	3 (23)	10 (77)	0
	Pancreatic tumor or cyst	15	4 (27)	11 (73)	0
	Pheochromocytoma	6	1 (17)	5 (83)	0
	CNS hemangioblastoma	5	1 (20)	4 (80)	0
Sunitinib ($n = 12$)	Renal cell carcinoma	15	6 (40)	5 (33)	4 (27)
	Renal cyst	4	1 (25)	3 (75)	0
	Pancreatic tumor or cyst	5	1 (20)	4 (80)	0
	CNS hemangioblastoma	2	0	2 (100)	0
	Pheochromocytoma	2	0	2 (100)	0
Sorafenib ($n = 11$)	Renal cell carcinoma	12	3 (25)	5 (42)	4 (33)
	Renal cyst	2	0	2 (100)	0
	Pancreatic tumor or cyst	5	2 (40)	3 (60)	0
	CNS hemangioblastoma	1	0	1 (100)	0
Axitinib ($n = 6$)	Renal cell carcinoma	6	2 (33)	4 (67)	0
	Renal cyst	5	1 (20)	4 (80)	0
	Pancreatic tumor or cyst	4	0	4 (100)	0
	CNS hemangioblastoma	2	1 (50)	1 (50)	0
	Pheochromocytoma	3	1 (33)	2 (67)	0
Pazopanib ($n = 3$)	Renal cell carcinoma	3	0	3 (100)	0
	Renal cyst	2	1 (50)	1 (50)	0
	Pancreatic tumor or cyst	1	1 (100)	0	0
	Pheochromocytoma	1	0	1 (100)	0

TKI, tyrosine kinase inhibitor; n, number of patients who participated in this treatment group; Total represents the response of all patients' lesions to TKIs.

TABLE 3 | Mean change in size compared to the baseline after TKI therapy.

Tumor type	Mean size (standard error)			*P*-value
	Baseline (cm)	After therapy (cm)	Percent change	
Renal cell carcinoma	3.79 (0.42)	3.06 (0.42)	−19.26	0.0001
Renal cyst	2.45 (0.39)	2.06 (0.34)	−15.92	0.027
Pancreatic tumor or cyst	2.6 (0.34)	2.12 (0.26)	−18.46	0.003
Hemangioblastoma	2.3 (0.59)	1.65 (0.40)	−28.26	0.073
Pheochromocytoma	3.82 (1.5)	3.25 (1.3)	−14.92	0.056

survival duration was 70 months for sunitinib-treated patients and 57 months in sorafenib-treated patients. Log-rank test results showed that overall survival ($P = 0.89$) and progression-free survival ($P = 0.44$) were not significantly different between the sunitinib- and sorafenib-treated patients (**Figure 2**).

We observed that nine patients achieved a favorable response, i.e., partial response, and eight patients showed progressive disease after TKI therapy. Comparisons of the clinical characteristics between the patients with a partial response and those with progressive disease are shown in **Table 4**. Type 1 VHL disease was found in all of the patients (8/8) in the progressive disease group and in 44.4% of the patients (4/9) in the partial response group (**Table 4**). Missense mutations were found in more than half (5/9) of the patients in the partial response group and in 25% (2/8) of the patients in the progressive disease group. In addition, the mean RCC diameter before TKI treatment was 3.2 cm in the partial response group, which was less than the mean diameter of 5.9 cm in the progressive disease group.

The most common side effects were hand–foot skin reactions, diarrhea, alopecia, hypertension, thrombocytopenia, and back pain in patients treated with one of the four TKIs (**Table 5**). These side effects were slight or mild in most patients, and severe side effects (grade 5) were not recorded. The prevalence of hand–foot skin reactions, diarrhea, and fatigue in sunitinib-treated patients was similar to that in previous studies of the side effects of sunitinib. The most common side effects were hand–foot skin reactions, diarrhea, alopecia, and thrombocytopenia in sorafenib-treated patients and hypertension, hand–foot skin reactions, and back pain in axitinib-treated patients. Other relatively rare side effects included periapical abscess (one case), perforation of the nasal septum due to repeat epistaxis (one case), severe hyperbilirubinemia (one case), hypertensive encephalorrhagia (one case with bilateral pheochromocytomas), and hypothyroidism (six cases). Most of these effects occurred in sunitinib-treated patients. Dose reduction was required

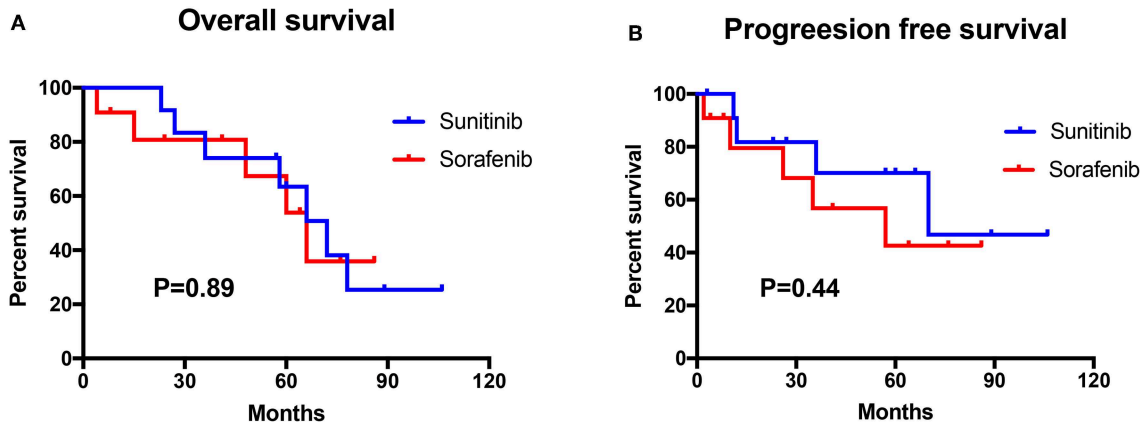

FIGURE 2 | Survival analysis of the sunitinib-treated group ($n = 12$) and the sorafenib-treated group ($n = 11$). **(A)** The overall survival (OS) between sunitinib and sorafenib treatment group. **(B)** The progression-free survival (PFS) between the sunitinib and the sorafenib treatment group.

in 13 (40.6%) of 32 patients, mainly in the sunitinib- and sorafenib-treated patients. The dose of sunitinib was reduced to 25–37.5 mg/day in six patients, and that of sorafenib was reduced to 400 mg/day in five patients. Dose reductions were rarely necessary in the axitinib- and pazopanib-treated patients.

DISCUSSION

This retrospective study on TKI therapy for patients with VHL disease in a single center showed that TKIs lessened the disease burden and that the side effects were acceptable. Primary data indicated a clinical benefit for patients with VHL disease with RCCs, pancreatic lesions, pheochromocytomas, renal cysts, and, possibly, CHBs (**Table 2**). The median duration of follow-up in this study was 31.5 months, which was longer than that in previous reports (17, 20). The longer follow-up period enabled us to observe the survival and TKI resistance of the patients. This is the largest retrospective study of TKIs in patients with VHL disease.

In this study, 32 patients were included, of whom 13 patients' mutation types were missense mutations, and the remaining were truncating mutations. Furthermore, we compared the landscape of VHL mutations with our previous study by Wang et al. and the international research from the Netherlands by Morgan Nordstrom-O'Brien et al. (**Supplementary Table 1**). We found that the landscape of VHL mutations was similar with previous reports (9, 26). Our study showed that many patients chose to continue TKI therapy because of the benefits of TKIs or the difficulties of surgical resection. Eleven (31%) of the 36 RCCs achieved partial response, 17 (47%) of 36 RCCs achieved stable disease, and 8 (22%) RCCs exhibited progressive disease (**Table 2**). Therapeutic benefits were also observed in pancreatic lesions and renal cysts. In 12 patients receiving TKI therapy for more than 3 years, we observed that pancreatic lesions and renal cysts were not cancerous and generally did not require treatment. Of the five CHBs, one achieved a partial response after 2 months of axitinib therapy, and of the six pheochromocytomas, 1 (17%) achieved a partial response after 6 months of axitinib therapy, which has rarely been reported in previous studies (21, 27).

Several pilot and retrospective studies have reported that sunitinib may be effective in patients with VHL disease, which is consistent with the results of our study. A phase 2 trial by Jonasch et al. reported that 6 (33%) of 18 RCCs showed partial response and that 19 (91%) of 21 HBs and all of the RAs and PNETs showed stable disease after 6 months of sunitinib treatment in 15 patients with VHL disease (17); common side effects included fatigue, diarrhea, anemia, and hand–foot skin reactions. In 2012, Ali et al. found that sunitinib was effective for PNET in patients with VHL disease (28). In addition, Kim et al. reported that metastatic RCCs treated with sunitinib exhibited a partial response that lasted for a long period of time in four patients with VHL disease (18). In our 12 patients with VHL disease treated with sunitinib, 6 (40%) of 15 RCCs and 1 (20%) of five pancreatic lesions showed a partial response (**Table 2**); common adverse reactions included hand–foot skin reactions, diarrhea, fatigue, and hypothyroidism.

Only one case report of sorafenib treatment for patients with VHL disease can be found in the literature. Choi et al. reported that low-dose sorafenib maintenance was an effective long-term treatment option for RCCs in patients with VHL disease who needed maximal preservation of renal function (22). We studied the effect of sorafenib in 11 patients with VHL disease, which is the largest retrospective study on sorafenib treatment for patients with VHL disease to date; 3 (25%) of 12 RCCs and 2 (40%) of five pancreatic lesions exhibited a partial response (**Table 2**), and the common adverse effects included hand–foot skin reactions, alopecia, diarrhea, and thrombocytopenia.

No report has examined axitinib treatment for patients with VHL disease. We assessed the effects and safety of axitinib in six patients with VHL disease and found that 2 (33%) of 6 RCCs, 1 of 3 pheochromocytomas, and 1 of 2 CHBs exhibited a partial response. Side effects mainly included hypertension, back pain, hand–foot skin reactions, and diarrhea. The sizes of CHBs were reduced after axitinib therapy in two patients. One CHB in the right cerebellum decreased from 2.3 × 1.7 cm to 1.9 × 1.4 cm on MRI, accompanied by significant alleviation of

TABLE 4 | Characteristics of patients between the PR group and the PD group.

Characteristic	Partial response group	Progressive disease group
No. of patients	9	8
Sex		
Male	4	5
Female	5	3
Family history		
Yes	7	7
No	2	1
Mutation Type		
Missense	5	2
Truncating	4	6
Clinical type		
Type 1	4	8
Type 2	5	0
Renal cell carcinoma		
Unilateral	2	0
Bilateral	7	8
Metastasis		
Yes	0	8
No	9	0
Survival		
Yes	8	1
No	1*	7
Mean RCC diameter before TKI therapy (cm)	3.2	5.9
Mean RCC diameter after TKI therapy (cm)	2	4.8
Mean age of RCC diagnosis (years)	35.7	41.1
Mean age of TKI treatment initiation (years)	36.3	46.5
Mean duration of TKI therapy (months)	44.9	37.3
Mean follow-up period (months)	56.3	49.6

*PR, partial response; PD, progressive disease; RCC, renal cell carcinoma, *patient who died after brain stem surgery.*

hydrocephalus and headache after 3 months of axitinib therapy, and another CHB in the L2–L3 spinal cord exhibited a partial response, with a decrease in size from 3.7 × 1.2 × 1.1 cm to 2.4 × 0.9 × 0.9 cm on MRI after axitinib treatment for 2 months. Therefore, axitinib may be effective for CHBs in patients with VHL disease. Larger prospective studies are warranted to further evaluate the efficacy and safety of axitinib in VHL-related CHBs.

Recently, a prospective study of pazopanib in patients with VHL disease by Jonasch et al. revealed that 13 (42%) of 31 patients achieved objective responses, and lesion site responses were observed in 31 (52%) of 59 RCCs, 9 (53%) of 17 pancreatic lesions, and 2 (4%) of 49 CHBs (20); side effects mainly included fatigue, diarrhea, transaminitis, and skin hyperpigmentation. These researchers suggested that pazopanib is effective for VHL disease and may be a treatment option for these patients. We treated three patients with pazopanib, and one RCC and one pancreatic lesion showed a partial response. Side effects mainly included diarrhea, back pain, and hand–foot skin reactions. Due to the small number of patients, we could not assess the efficacy of pazopanib in patients with VHL disease.

In this study, the renal cyst includes renal simple cyst and renal complex cyst. Although the response of renal cyst of VHL disease to TKIs has not been reported in previous research, we found that some TKIs were used to treat autosomal-dominant polycystic kidney disease (ADPDK) in previous literature; the research considered that TKIs can be used to decrease EGFR tyrosine kinase activity and collecting tubule cyst formation and enlargement in polycystic kidney disease (29, 30). In our study, we found 3 (23%) of 13 renal cyst responses to TKIs. We think that the results may be attributed to renal complex cyst, as the previous article revealed that complex cystic and solid lesions can contain neoplastic tissue that frequently enlarges (3). We think that the neoplastic tissue of renal complex cyst may respond to TKIs therapy, which was consistent with the observations in our clinical practice. Larger prospective studies should be performed to further evaluate the efficacy of TKIs in VHL-related renal cyst.

The reason why organ-specific VHL-derived tumors respond differently to TKIs is unclear until now. We know that VHL disease is a multi-organ cancer syndrome, which is characterized by the development of several benign or malignant tumors and cysts in many organ systems. A previous study by Kluger HM et al. showed that VEGF and VEGF receptors were tightly co-expressed in human RCCs specimen ($P < 0.001$) (31). In 2011, a phase 2 trial by Jonasch et al. found that VEGFR2, pVEGFR2, and phosphorylated-to-total VEGFR2 ratios were statistically significantly higher in the RCC than in the HB samples ($P = 0.001$), and their study reported that 6 of 18 RCCs (33%) responded partially to sunitinib, vs. none of 21 HBs ($P = 0.014$) (17). In our study, RCC shows a higher partial response rate for TKIs compared to other VHL-related tumors. We know that sunitinib, sorafenib, axitinib, and pazopanib are small-molecule inhibitors of vascular endothelial growth factor receptors (VEGFRs) (16, 17, 32). Therefore, we think that tumor-specific genetic lesions or tissue-specific endothelial heterogeneity may explain these differences in response. Future studies will be conducted to further explain the reason why organ-specific VHL-derived tumors respond differently to TKIs.

During TKI therapy, a periapical abscess occurred in one case during the sixth cycle of sunitinib therapy, and the drug was discontinued for 17 weeks to treat the periapical abscess. This patient also presented hypothyroidism in the ninth cycle of therapy, and Euthyrox was administered once per day. Hypothyroidism and perforation of the nasal septum due to repeated epistaxis were observed in one patient. In this study, hypothyroidism was observed in six patients, of whom four were administered Euthyrox during TKI therapy. Among the patients with hypothyroidism, 5 (41.7%) cases occurred in sunitinib-treated patients, which is consistent with

TABLE 5 | Treatment-emergent toxic effects.

Toxicity	All grades	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Sunitinib group	Sorafenib group	Axitinib group	Pazopanib group
Hand–foot skin reaction	20	9	8	3	0	0	7	8	3	2
Diarrhea	19	11	6	2	0	0	8	6	2	3
Alopecia	13	11	2	0	0	0	4	7	1	1
Hypertension	12	5	3	4	0	0	4	3	4	1
Thrombocytopenia	10	7	3	0	0	0	4	5	1	0
Pain	10	5	3	2	0	0	3	2	3	2
Fatigue	8	4	3	1	0	0	6	1	1	0
Hyperbilirubinemia	7	4	2	0	1	0	4	3	0	0
Hypothyroidism	6	3	3	0	0	0	5	0	1	0
Dysgeusia	5	3	2	0	0	0	4	0	0	1
Transaminitis	5	3	2	0	0	0	0	4	0	1
Mucositis	4	3	1	0	0	0	3	0	0	1
Rash	4	3	1	0	0	0	1	3	0	0
Anorexia	4	2	2	0	0	0	4	0	0	0
Nausea	4	2	2	0	0	0	2	1	0	1
Anemia	3	3	0	0	0	0	0	2	0	1
Menolipsis	3	3	0	0	0	0	2	0	1	0
Vomiting	3	2	1	0	0	0	0	2	1	0
Headache	3	2	1	0	0	0	2	0	1	0
Hyperuricemia	2	2	0	0	0	0	1	1	0	0
Infection	2	2	0	0	0	0	2	0	0	0
Dyspnea	1	1	0	0	0	0	0	1	0	0
Edema (head/neck)	1	1	0	0	0	0	1	0	0	0
Elevated creatinine	1	1	0	0	0	0	0	0	0	1
Hydropericardium	1	1	0	0	0	0	1	0	0	0
Epistaxis	1	0	0	1	0	0	1	0	0	0
Encephalorrhagia	1	0	0	0	1	0	1	0	0	0

the results of previous studies (33). Previous prospective studies indicate that sunitinib can induce hypothyroidism in 36–71% of patients. Hyperbilirubinemia was observed in one patient, who required hospitalization after 15 days of sunitinib therapy. One patient with bilateral giant pheochromocytomas (L: 7.9 × 7.4 cm; R: 8.6 × 4.7 cm) was recommended to undergo TKI treatment primarily because of the risks during surgical resection. Hypertensive encephalorrhagia occurred in one patient after drug discontinuance during the 52nd treatment cycle, and sunitinib treatment was resumed after partial recovery from the cerebral hemorrhage sequelae.

This was a retrospective study, and thus, information bias may exist. In addition, only six patients were treated with axitinib, and three patients were treated with pazopanib, which limited our evaluation of the value of axitinib and pazopanib for patients with VHL disease.

In conclusion, this research is the largest retrospective study of TKIs in patients with VHL disease. Our results showed that TKIs were partially effective for RCCs, pancreatic lesions, and pheochromocytomas, and possibly effective for CHBs, and the side effects were acceptable. Further evaluation of TKIs in patients with VHL disease in larger prospective studies is warranted.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The name of the repository and accession number(s) are not available.

ETHICS STATEMENT

This study was approved by the Medical Ethics Committee of Peking University First Hospital (Beijing, China). Informed consent was obtained from patients or their legal guardians.

AUTHOR CONTRIBUTIONS

KG and LC developed the hypothesis and secured funding. KM wrote the first draft of the paper. JW, SL, XP, BZ, and JZha carried out statistical analyses. JZho, BH, HX, KZ, and LL dealt with figures and tables. YG, DC, and ZW performed data collection. BH revised the manuscript. All authors critically commented on and approved the final submitted version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01122/full#supplementary-material

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Genomic Features and Clinical Characteristics of Adolescents and Young Adults With Cholangiocarcinoma

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Background: Adolescents and young adults (AYAs) diagnosed with cancer between ages 15 and 45 years may exhibit unique biologic and genomic characteristics as well as clinical features, resulting in differences in clinical characters and drug resistance. However, compared to other solid cancers, relatively few studies have been conducted in this age group in cholangiocarcinoma (CCA). This study is performed to investigate the clinical and molecular features of AYAs with CCA.

Methods: Three cohorts, including the external dataset (TCGA and MSKCC) and the perihilar CCA databank of Chinese tertiary hospitals, were contained in this study. Pathway and process enrichment analysis had been carried out with the following ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, and CORUM. Metascape and GEPIA datasets were used for bioinformatic analysis. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism (version 7.0; GraphPad Software, La Jolla, California) and R studio (version 3.6.1; R studio, Boston, Massachusetts).

Results: Compared to older adults, AYAs with CCA presented with worse overall survival, although the difference was not significant. Specific to patients with stage IV CCAs who underwent chemotherapy, AYAs were associated with significantly poorer overall survival (OS) ($p = 0.03$, hazards ratio (HR) 3.01, 95% confidence interval (CI) 1.14-4.91). From the anatomical perspective, more extrahepatic CCA was detected in the AYA group. Microsatellite instability (MSI) occurred in 3% of older patients in the present study. Nevertheless, none of the AYAs had MSI status. In this study, AYAs gained an enhanced frequency of additional sex combs like 1 (ASXL1) ($p = 0.02$) and KMT2C ($p = 0.02$) mutation than their older counterparts. Besides ASXL1 and KMT2C, the genes enriched in AYAs with CCA were analyzed by pathway and process enrichment analysis. And those genes were found to be associated with poorer differentiation, deubiquitination, and WNT signal pathway. Moreover, AYAs were relevant to poor differentiation and advanced tumor stage.

Conclusion: This study offered a preliminary landscape of the clinical and molecular features of early-onset biliary cancers. Further studies including more samples are essential to investigate whether ASXL1 and KMT2C could be considered as potentially targetable genomic signatures for young patients.

Keywords: adolescents and young adults (AYAs), mutation, cholangiocarcinoma, early-onset, ASXL1

INTRODUCTION

Cholangiocarcinoma (CCA) is a highly fatal malignant tumor with rising incidence. It accounts for ∼10–25% of all hepatobiliary malignancies and <1% of all types of cancers (1). The incidence of adolescents and young adults (AYAs) with CCA was even less. Despite recognition of the importance of AYAs with cancers, the biologic and genomic characteristics of AYAs with CCA remain largely unknown.

AYAs diagnosed with cancer between ages 15 and 45 years may exhibit unique biologic and genomic features, resulting in differences in clinical behaviors and chemotherapy/targeted therapy resistance (2). These features could also be clinically exploited to develop companion diagnostics and novel therapies for treating AYAs with cancers (3). For instance, AYAs with solid tumors, such as colorectal carcinomas, are more likely to exhibit signet-ring histology, synchronous or metachronous metastasis, and present at a late stage (4, 5). From the mutational perspective, most early-onset (age <50 years) patients present with lower prevalence of KRAS, BRAF, and NRAS mutations in comparison with late-onset patients (6).

To date, AYAs with other solid tumors have been extensively described in the literature. However, few studies have been conducted for patients with CCA at this age group. Despite, most recently, genomic analysis of patients with CCA being performed by the Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering Cancer Center (MSKCC) (7), the genomic underpinnings of these AYAs with this rare cancer remain largely unknown. Therefore, in this study, the clinical and molecular features of AYA CCA patients were investigated by analyzing the external dataset (8, 9) and internal hilar CCA databank to shed light on early-onset biliary malignancy.

METHODS

Study Population and Data Collection

Three cohorts were included in the present study. The first cohort included 155 consecutive patients with perihilar CCA (pCCA) from three hepatobiliary surgery centers affiliated to tertiary hospitals in China between January 2013 and November 2018. Eighteen patients (12%) in this cohort were AYA (aged 15–45 years) and were set as *AYA group*. The rest (age >45) was set as the group "*Others*." This retrospective study was approved by the institutional review board (IRB) of the Renji Hospital and the Study Group of Biliary Surgery of the surgical branch of the Chinese Medical Association.

In the second cohort, the genomic data (e.g., mutation frequency) of AYAs and the elderly with CCAs extracted from the TCGA database were compared. This cohort included five AYA (10%) and 46 elder patients.

The third cohort contained the data of age-associated gene mutation of 192 patients with CCA extracted from the MSKCC dataset, including 26 (14%) AYAs. cBioPortal platform (www.cbioportal.org) was used for analyzing (8, 9) (**Table 1**).

Follow-Up

In the present study, progression-free survival (PFS) was defined as the time after the treatment with the disease not getting worse. Disease-free survival (DFS) was the time for any recurrence. If the postoperative margin was negative, the operation was considered as R_0 resection. Follow-up consisted of serum tumor marker measurements every 1–3 months and computed tomography (CT) every 6 months. Complete follow-up was conducted for the entire cohort of patients.

Pathological Evaluation

Tumor specimens were sent for pathological evaluation about the quality, grading, tumor stage according to AJCC 7th edition, risk factor (perineuronal invasion, etc.), and lymph node status. CCAs are a heterogeneous group of tumors that can be classified into three clinically distinct types of cancers, intrahepatic CCA (iCCA), pCCA, and distal CCA (dCCA) basing on its anatomical location. pCCA and dCCA were also grouped as extrahepatic CCA (eCCA). Specifically, pCCA in the present study was defined as the CCA that developed at the point where the left and right hepatic ducts joined to form the common hepatic duct by imaging (CT or magnetic resonance cholangiopancreatography).

MSI/MSS Status and TMB Evaluation

Programmed cell death protein 1 (PD-1) blockade provides a therapeutic opportunity for patients with high tumor mutation burden (TMB), high microsatellite instability (10) (MSI-H), and deficient mismatch repair (dMMR). Therefore, the MSI score, microsatellite instability (MSI)/microsatellite stability (MSS) status, and TMB were also analyzed between the two groups by using cBioPortal platform.

Perioperative Evaluation

The intraoperative evaluation included the length of operation, intraoperative hemorrhage, intraoperative blood transfusion, and vascular anastomosis. Additionally, blood routine examination, biochemical test, total bilirubin (Blood) (TBil), aspartate transaminase (AST), alanine transaminase (ALT), and so on, and other hepatic and renal function examinations were performed perioperatively.

TABLE 1 | The clinical character of AYA patients and older patients.

	AYA (<=45)		Others (>45)	
Sample size	26		166	
Gender (male:female)	12:14		88:78	
Age (year)*	40 (26–45)		64 (46–87)	
Stage IV	15	58%	112	68%
Recurrence				
Recurrence	6	23%	36	22%
Non-recurrence	4	15%	4	2%
Not applicable	17	65%	125	75%
Metastatic site	7	27%	43	26%
Liver	3	12%	12	7%
Lung	0	0%	4	2%
Lymph node	0	0%	13	8%
Brain	1	4%	0	0%
Omentum	0	0%	3	2%
Peritoneum	1	4%	6	4%
Pleura	2	8%	0	0%
Pelvis	0	0%	1	1%
Others	0	0%	4	2%
MSI score	0.88	(0–5.11)	0.94	(0–35.01)
TMB score	4.84	(2–17.7)	4.26	(1–47.2)
Systematic therapy	18	69%	138	83%
FOLFOX	2	11%	7	5%
FOLFIRINOX	2	11%	0	0%
Gemcitabine	2	11%	3	2%
Gem/cis	10	56%	64	46%
GemOX	2	11%	22	16%
Bevacizumab/FUDR	0	0%	1	1%
Cape/OX	0	0%	1	1%
Capecitabine	0	0%	1	1%
FUDR/GemOX	0	0%	9	7%
Gax	0	0%	1	1%
Gem/abraxane	0	0%	2	1%
Gem/Cape	0	0%	3	2%
Gem/Cis/MEK162	0	0%	18	13%
Gem/erlotinib	0	0%	1	1%
Gem/taxol	0	0%	1	1%
G-FLIP	0	0%	1	1%
Irinotecan + HAI FUDR	0	0%	1	1%
Sorafenib	0	0%	1	1%
TDM-1	0	0%	1	1%

*$p < 0.05$; **$p < 0.01$.

Enrichment Analyses

Metascape (http://metascape.org/gp/index.html) is an effective and efficient tool for experimental biologists to comprehensively analyze and interpret OMICs-based studies in the big data era (19). The database was used to perform the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, which is used to predict the potential biological functions of the overlapping genes of the DEGs and target genes. Then, verification was performed by the GEPIA database (http://gepia.cancer-pku.cn) to identify hub genes (11–19).

Statistical Analysis

Pearson's Chi-square test for categorical variables and the Wilcoxon rank-sum test for continuous variables were used to compare various parameters in AYA and the other group. The Kaplan-Meier method was used to estimate overall survival (OS), DFS, or PFS. Differences in survival outcomes were assessed by the log-rank test. Results were presented as hazard ratios (HRs) and 95% confidence intervals (CIs). $P < 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism (version 7.0; GraphPad Software, La Jolla, California) and R studio (version 3.6.1; R studio, Boston, Massachusetts).

RESULTS

Clinicopathologic Features of AYAs With CCA

From the prognosis perspective, the length of OS in AYAs with CCA was worse (36 vs. 44 months) than the older patients. However, the difference was not significant (**Figure 1A**; $p = 0.26$, HR 1.39, 95% CI 0.78–2.47). Specific to patients with stage IV CCAs who underwent chemotherapy, AYAs were associated with significantly poorer OS (**Figure 1B**; $p = 0.03$, HR 3.01, 95% CI 1.14–4.91), and the survival period was almost half of their older counterparts (18 vs. 34 months). From the anatomical perspective, more eCCA was detected in the AYA group (29 vs. 17%, **Figure 1C**).

Molecular Features of AYAs With CCA

PD-1 blockade provides a therapeutic opportunity for patients with high TMB, MSI-H, and dMMR. Therefore, the MSI score, MSI/MSS status, and TMB (**Figure 1D**) were also analyzed between the two groups. It has been reported that MSI status occurred in 3–10% of CCA; consistently, MSI occurred in 3% of older patients (>45 years old) in the present study. Intriguingly, none of the AYA patients had MSI status, although the average MSI score was similar (**Figure 1E**; AYA group: 0.8785 ± 0.2727, Others group: 0.944 ± 0.2831) between the two groups. Additionally, AYA patients had similar TMB compared to their counterparts (AYA group: 4.258 ± 0.3885, Others group: 4.452 ± 0.8883).

Somatic Mutations of CCA in AYA Patients

Additional sex combs like 1 (ASXL1) is the obligate regulatory subunit of a deubiquitinase complex. Heterozygous mutations of ASXL1 are frequent in myeloid leukemias and other malignancies. Here we demonstrated in the first cohort that AYAs with CCAs gained a higher frequency of ASXL1 mutation than their older counterparts [**Figure 1F**; $p = 0.02$, 11% (3/27) vs. 1% (2/167)].

KMT2C mutates frequently and is considered crucial for the occurrence and development of numerous cancers. In the present study, significantly higher KMT2C (histone lysine

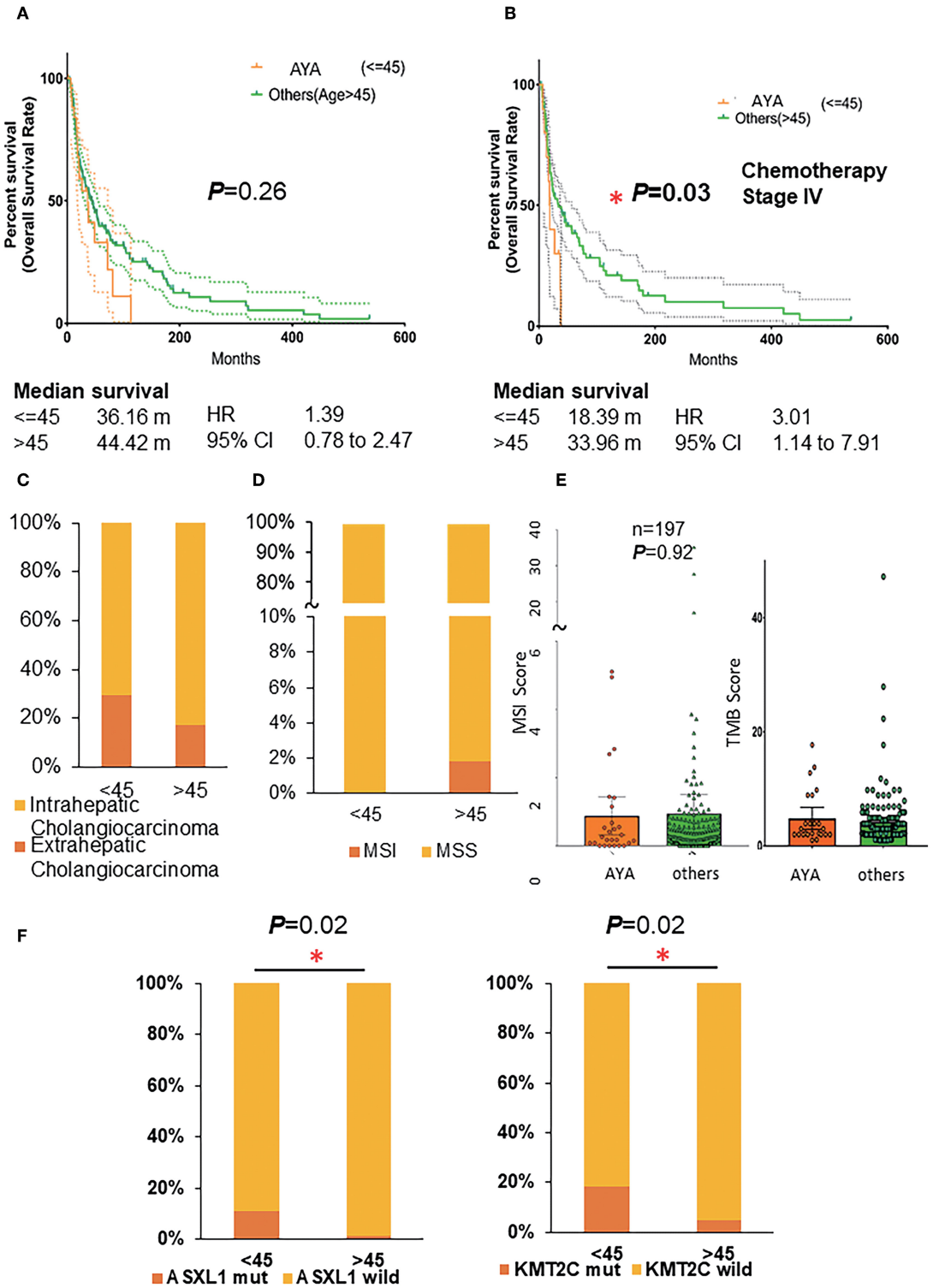


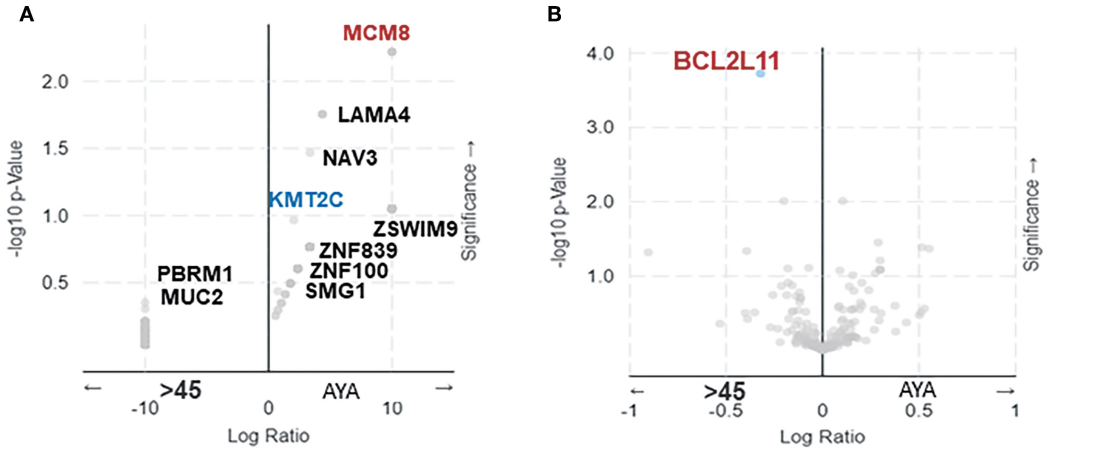
FIGURE 1 | (A) Overall survival rate of AYA patients and others (age >45). **(B)** Overall survival rate of AYA patients and others (age >45) with stage IV cholangiocarcinoma and underwent the treatment of chemotherapy. **(C)** The proportion of intrahepatic and extrahepatic cholangiocarcinoma in AYA (<=45) and other (>45) groups. **(D)** The MSI/MSS status of patients in AYA (<=45) and other(>45) groups. **(E)** The MSI score and TMB score of patients in AYA (<=45) and other (>45) groups. **(F)** The mutation frequency of ASXL1 and KMT2C of patients in AYA (<=45) and other (>45) groups basing on cohort 3 (MSKCC). AYA, adolescents and young adults; MSI, microsatellite instability; TMB, tumor mutation burden.

TABLE 2 | Comparison of genes with different mutation frequency in both groups.

Gene	Cytoband	(A) AYA	(B) OA	*p*-value	Gene	Cytoband	(A) AYA	(B) OA	*p*-value
ASXL1	20q11.21	3 (11.11%)	2 (1.19%)	0.0197	EP300	22q13.2	1 (3.70%)	1 (0.60%)	0.258
KMT2C	7q36.1	5 (18.52%)	8 (4.76%)	0.0206	ERBB4	2q34	1 (3.70%)	1 (0.60%)	0.258
ERBB3	12q13.2	3 (11.11%)	4 (2.38%)	0.0569	FLT4	5q35.3	1 (3.70%)	1 (0.60%)	0.258
FAT1	4q35.2	2 (7.41%)	2 (1.19%)	0.093	FOXA1	14q21.1	1 (3.70%)	1 (0.60%)	0.258
SOX9	17q24.3	2 (7.41%)	2 (1.19%)	0.093	KIAA1217	10p12.2-p12.1	1 (3.70%)	1 (0.60%)	0.258
KRAS	12p12.1	1 (3.70%)	22 (13.10%)	0.136	MALT1	18q21.32	1 (3.70%)	1 (0.60%)	0.258
AR	Xq12	1 (3.70%)	0 (0.00%)	0.138	MEN1	11q13.1	1 (3.70%)	1 (0.60%)	0.258
AXIN2	17q24.1	1 (3.70%)	0 (0.00%)	0.138	MST1R	3p21.31	1 (3.70%)	1 (0.60%)	0.258
CDKN1A	6p21.2	1 (3.70%)	0 (0.00%)	0.138	NCOA3	20q13.12	1 (3.70%)	1 (0.60%)	0.258
DICER1	14q32.13	1 (3.70%)	0 (0.00%)	0.138	TCF3	19p13.3	1 (3.70%)	1 (0.60%)	0.258
FGFR4	5q35.2	1 (3.70%)	0 (0.00%)	0.138	TSC2	16p13.3	1 (3.70%)	1 (0.60%)	0.258
GATA2	3q21.3	1 (3.70%)	0 (0.00%)	0.138	ZFHX3	16q22.2-q22.3	1 (3.70%)	1 (0.60%)	0.258
GNA11	19p13.3	1 (3.70%)	0 (0.00%)	0.138	PIK3CA	3q26.32	3 (11.11%)	10 (5.95%)	0.261
GRIN2A	16p13.2	1 (3.70%)	0 (0.00%)	0.138	BAP1	3p21.1	2 (7.41%)	24 (14.29%)	0.262
HIST3H3	1q42.13	1 (3.70%)	0 (0.00%)	0.138	TERT	5p15.33	0 (0.00%)	8 (4.76%)	0.296
JAK1	1p31.3	1 (3.70%)	0 (0.00%)	0.138	TP53	17p13.1	8 (29.63%)	39 (23.21%)	0.307
LAMC1	1q25.3	1 (3.70%)	0 (0.00%)	0.138	SMAD4	18q21.2	1 (3.70%)	15 (8.93%)	0.317
MDM2	12q15	1 (3.70%)	0 (0.00%)	0.138	ATM	11q22.3	1 (3.70%)	14 (8.33%)	0.354
NOL4	18q12.1	1 (3.70%)	0 (0.00%)	0.138	KMT2D	12q13.12	2 (7.41%)	7 (4.17%)	0.361
PDCD1	2q37.3	1 (3.70%)	0 (0.00%)	0.138	APC	5q22.2	1 (3.70%)	2 (1.19%)	0.362
PHOX2B	4p13	1 (3.70%)	0 (0.00%)	0.138	ATR	3q23	1 (3.70%)	2 (1.19%)	0.362
PLK2	5q11.2	1 (3.70%)	0 (0.00%)	0.138	BRD4	19p13.12	1 (3.70%)	2 (1.19%)	0.362
RABGAP1L	1q25.1	1 (3.70%)	0 (0.00%)	0.138	GATA1	Xp11.23	1 (3.70%)	2 (1.19%)	0.362
RASAL2	1q25.2	1 (3.70%)	0 (0.00%)	0.138	IGF1R	15q26.3	1 (3.70%)	2 (1.19%)	0.362
SOX17	8q11.23	1 (3.70%)	0 (0.00%)	0.138	KDM5A	12p13.33	1 (3.70%)	2 (1.19%)	0.362
STAG2	Xq25	1 (3.70%)	0 (0.00%)	0.138	PTCH1	9q22.32	1 (3.70%)	2 (1.19%)	0.362
TACC2	10q26.13	1 (3.70%)	0 (0.00%)	0.138	XPO1	2p15	1 (3.70%)	2 (1.19%)	0.362
TGFBR2	3p24.1	1 (3.70%)	0 (0.00%)	0.138	DOT1L	19p13.3	0 (0.00%)	6 (3.57%)	0.404
ARID1B	6q25.3	2 (7.41%)	3 (1.79%)	0.142	CDH1	16q22.1	1 (3.70%)	3 (1.79%)	0.452
CTNNB1	3p22.1	2 (7.41%)	3 (1.79%)	0.142	EPHA5	4q13.1-q13.2	1 (3.70%)	3 (1.79%)	0.452
KMT2A	11q23.3	2 (7.41%)	3 (1.79%)	0.142	IDH2	15q26.1	1 (3.70%)	3 (1.79%)	0.452
FGFR2	10q26.13	5 (18.52%)	16 (9.52%)	0.144	MAP2K1	15q22.31	1 (3.70%)	3 (1.79%)	0.452
PBRM1	3p21.1	5 (18.52%)	16 (9.52%)	0.144	MAP3K1	5q11.2	1 (3.70%)	3 (1.79%)	0.452
SMARCA4	19p13.2	2 (7.41%)	4 (2.38%)	0.195	POLE	12q24.33	1 (3.70%)	3 (1.79%)	0.452
NRAS	1p13.2	2 (7.41%)	5 (2.98%)	0.25	SETD2	3p21.31	1 (3.70%)	3 (1.79%)	0.452
ASXL2	2p23.3	1 (3.70%)	1 (0.60%)	0.258	ARID2	12q12	0 (0.00%)	5 (2.98%)	0.471
CARD11	7p22.2	1 (3.70%)	1 (0.60%)	0.258	ARID1A	1p36.11	5 (18.52%)	35 (20.83%)	0.507
CREBBP	16p13.3	1 (3.70%)	1 (0.60%)	0.258	IDH1	2q34	7 (25.93%)	41 (24.40%)	0.516
EIF4A2	3q27.3	1 (3.70%)	1 (0.60%)	0.258					

methyltransferase 2C) mutation rate was in the AYA group [**Figure 1F**; $p = 0.02$, 19% (5/27) vs. 4.7% (8/169)]. Specifically, 40% of the patients who had mutated ASXL1 also harbored a mutated KMT2C (also known as MLL3), KMT2D, or ARID1A. And 38.5% of the KMT2C mutated synergistically with ARID1A mutation. Additionally, although the difference was not significant, AYAs were likely to harbor more frequent mutated FGFR2 (18.5 vs. 9.5%) or PBRM1 (18.5 vs. 9.5%) or ERBB3 (11.1 vs. 2.4%) genes and less BAP1, KRAS, and SMAD4 (**Supplemental Figures 1A,B**; **Table 2**).

In the second cohort extracted from the TCGA dataset, the MCM8 gene mutation ($p < 0.05$) was significantly enriched in AYAs with CCA. Besides KMT2C, mutations of LAMA4, AGAP6, AKAP13, ARMC12, MAP1A, NAV3, ADAMTS7, FTH1, and ITPR2 were also observed in AYAs with CCA (**Figure 2A**). From the protein expression aspect, BCL2L11 was significantly downregulated in AYAs (**Figure 2B**; $q = 0.0383$). From the RNA expression perspective, PIK3C3, IQCH, RGP1, and LPP were upregulated in the AYA group (**Supplemental Figure 1C**).



Mutation frequency

Gene	Cytoband	Young On-set	Others (>45)	p-Value	Enriched in
AGAP6	10q11.23	50%	0 %	6.06E-03	AYA
AKAP13	15q25.3	50%	0 %	6.06E-03	AYA
ARMC12	6p21.31	50%	0 %	6.06E-03	AYA
MAP1A	15q15.3	50%	0 %	6.06E-03	AYA
MCM8	20p12.3	50%	0 %	6.06E-03	AYA
ADAMTS7	15q25.1	50%	2.4%	0.0176	AYA
FTH1	11q12.3	50%	2.4%	0.0176	AYA
ITPR2	12p11.23	50%	2.4%	0.0176	AYA
LAMA4	6q21	50%	2.4%	0.0176	AYA
NAV3	12q21.2	50%	4.9%	0.0341	AYA

Protein expression (RPPA)

Gene	Cytoband	p-Value	q-Value	Enriched in
BCL2L11	2q13	1.89E-04	0.0383	>45
ANXA7	10q22.2	9.76E-03	0.66	AYA
YBX1	1p34.2	9.80E-03	0.66	>45
BRAF	7q34	0.0353	0.992	AYA
EIF4G1	3q27.1	0.0411	0.992	AYA
PXN	12q24.23	0.0427	0.992	AYA
CAV1	7q31.2	0.0459	0.992	>45
FN1	2q35	0.048	0.992	>45

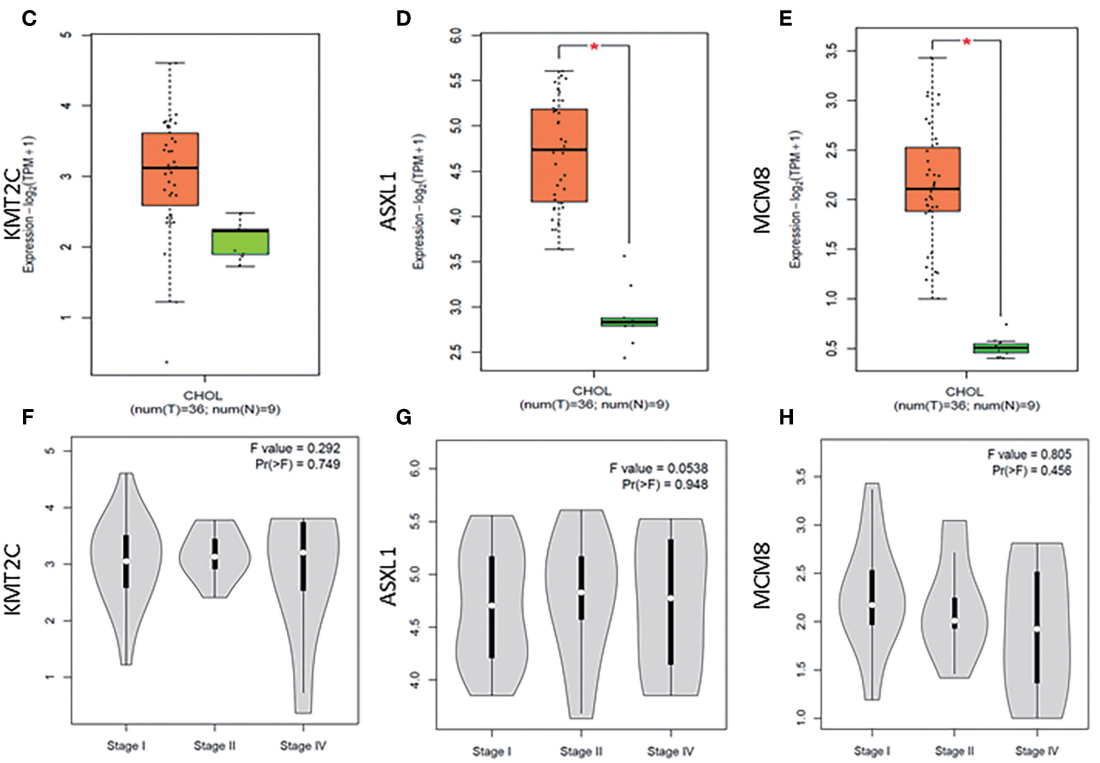


FIGURE 2 | (A) The mutation frequency of presentative genes ($p < 0.05$) in AYA and other groups basing on cohort 2. **(B)** The difference of protein expression between the two groups basing on cohort 2. **(C–E)** The expression level of ASXL1, KMT2C, and MCM8 in tumor vs. paired normal samples in CCA. **(F–H)** Expression level of ASXL1, KMT2C, and MCM8 in different tumor stages. AYA, adolescents and young adults; ASXL1, additional sex combs like 1. *$P < 0.05$.

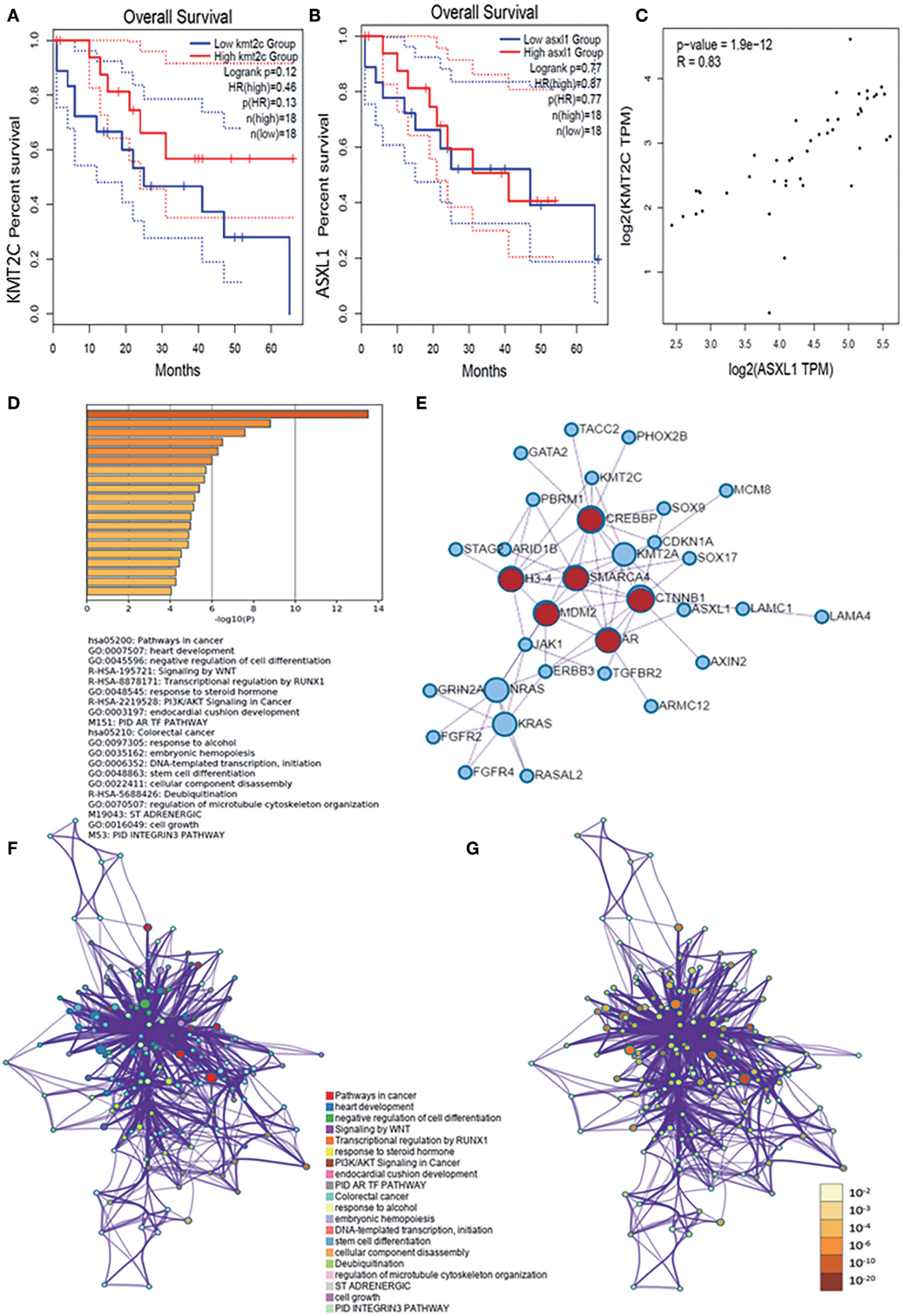


FIGURE 3 | (A,B) Survival analysis based on the expression status of KMT2C and ASXL1 and a Kaplan-Meier curve was plotted. **(C)** Correlations of KMT2C and ASXL1 in CCA. **(D)** Bar graph of enriched terms across these enriched genes in AYAs with CCA, colored by *p*-values. **(E)** Protein–protein interaction network and MCODE components identified in the genes enriched in AYAs with CCA. **(F,G)** Network of enriched terms: **(F)** colored by cluster-ID, where nodes that share the same cluster ID are typically close to each other; **(G)** colored by *p*-value, where terms containing more genes tend to have a more significant *p*-value. ASXL1, additional sex combs like 1; AYA, adolescents and young adults; CCA, cholangiocarcinoma.

Overexpression of KMT2C and ASXL1 in CCA

We then verified the expression level of KMT2C, ASXL1, and MCM8 in CCA using the GEPIA database and found that all of the three genes, especially ASXL1 ($p < 0.05$) and MCM8 ($p < 0.05$), were overexpressed in tumor tissues (**Figures 2C–E**). However, the expression level of the three genes was associated with neither tumor stages nor OS rate, respectively (**Figures 2F–H**, **3A,B**). Pearson's correlation coefficient of ASXL1 and KMT2C was 0.83 (**Figure 3C**).

Pathway and Process Enrichment Analysis of the Enriched Genes in AYAs

For these enriched genes in AYAs with CCA, pathway and process enrichment analysis had been carried out with the following ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, and CORUM. Top 20 clusters with their enriched representative terms were shown in **Figure 3D**. To further capture the relationships between the terms, a subset of enriched terms had been selected and rendered as a network plot, where terms with a similarity >0.3 were connected by edges. The network was visualized using Cytoscape, where each node represented an enriched term and was colored first by its cluster ID (**Figure 3F**) and then by its *p*-value (**Figure 3G**). Specifically, the genes enriched in AYAs with CCA were associated with several pathways, such as cancer-associated pathways, negative regulation of cell differentiation, deubiquitination, WNT signal pathway, and so on.

Then, for these enriched genes in AYAs with CCA, protein–protein interaction enrichment analysis had also been carried out. Densely connected network components, including MDM2, SMARCA4, CTNNB1, AR, CREBBP, H3-4, were identified in **Figure 3E**.

Clinical Characters and Postoperative Prognosis of AYAs With pCCA

External genomic profiles (cohort 2, cohort 3) were analyzed, and it was found that iCCA presented significant better OS than eCCA ($p = 0.04$, 44 vs. 35 months) and slightly better than pCCA, too ($p = 0.09$, 40 vs. 18 months) (**Figures 4A,B**).

As is known, for patients in the intrahepatic, perihilar, and distal groups, the 5-year survival was 40, 10, and 23%, respectively (20). The prognosis of pCCA was the worst. Thus, by using our pCCA dataset containing 245 patients, we further investigated the prognosis between AYAs (cohort 1) and older patients (>45). Intriguingly, these patients had similar PFS (**Figure 4C**; $p = 0.73$, 15 vs. 15 months, HR 1.12, 95% CI 0.58–2.14) and OS rate (**Figure 4D**; $p = 0.84$, 34 vs. 15 months, HR 0.92, 95% CI 0.42–2.00).

Moreover, it was shown that AYAs were relevant to poor differentiation (**Figure 4E**) and advanced tumor stage (III and IV, 67%, **Figure 4E**). All AYAs in the current study presented with moderate and poor differentiation (**Table 1**). The comparison of chemical examinations showed that TBil value of older patients (>45 years old) were significantly elevated (**Figure 4F**).

DISCUSSION

Recognition of the clinical and genomic characters of AYAs with CCA is crucial for treatment strategy design. The treatments, especially targeted therapy and immunotherapy of AYAs, may differ from those best suited to older patients. It was reported that solid cancers (21), such as colorectal carcinoma, in AYAs were more aggressive and associated with a poorer prognosis as well as enriched MSI-H status compared to older patients (22, 23). In contrast, no MSI status was detected in AYAs with CCA in the present study. In the older patients' group, MSI occurred in 3∼10% of the patients, similar to the reported general probability in all CCAs. The length of survival of AYAs (1.5 years) was almost half of the older patients (3 years); however, owing to the small sample size, no statistical significance was achieved. This was also the limitation of the present study.

The present study provided an initial landscape of genes that displays a greater mutational frequency in AYAs with CCA. Specifically, ASXL1 and KMT2C were found more frequent in AYAs compared with older patients with CCA.

ASXL1 mutations were known to be upregulated in solid cancers with metastasis (24) and in castration-resistant prostate cancer (CRPC) (25). Intriguingly, the significantly greater mutation frequency of ASXL1 combined with lower KRAS mutation was reported in kinase rearrangements (KRE). And lower KRAS mutation frequency was also detected in AYA patients as reported. Moreover, the high mutation rate of ASXL1 rates was also associated with MSI status enrichment (26). In the present study, the mutation frequency of ASXL1 was significantly higher in AYAs. KRAS mutation also tended to decrease but without statistical significance owing to the inadequate sample size. The only inconsistency was that all AYAs with CCA had MSS status instead of MSI. Patients with MSI-H status and KRE could benefit from both tyrosine kinase inhibitor (TKI) and checkpoint inhibitor treatment. However, this advantage seems to attenuate in AYAs with CCA. In contrast, it was reported that the transcription regulator ASXL1 mutation was associated with poorer outcomes as well as drug resistance (27), which might explain why AYAs with stage IV CCAs who underwent chemotherapy had worse prognosis in the present study.

Similar to ASXL1, KMT2C mutation was also enriched in late-stage or metastatic status of iCCA (28), breast cancer (29), and prostate cancer (30) and was associated to poor prognosis (31). Especially in AYAs with late-stage CCA, greater ASXL1 and KMT2C mutation rates were detected, which might suggest that CCAs in AYA patients is more aggressive.

Besides ASXL and KMT2C, the genes enriched in AYAs with CCA were analyzed by pathway and process enrichment analysis. And those genes were found to be associated with poorer differentiation, deubiquitination, and WNT signal pathway. Surgical resection remains the mainstay of potentially curative treatment for CCA. However, the probability of radical curative resection is low, and the prognosis is insufficient. Molecular profiling has delineated the genomic and transcriptomic characters of each CCA subtype. However, the genomic signature of AYA patients was not reported before. This study offered a preliminary landscape of the clinical and molecular features

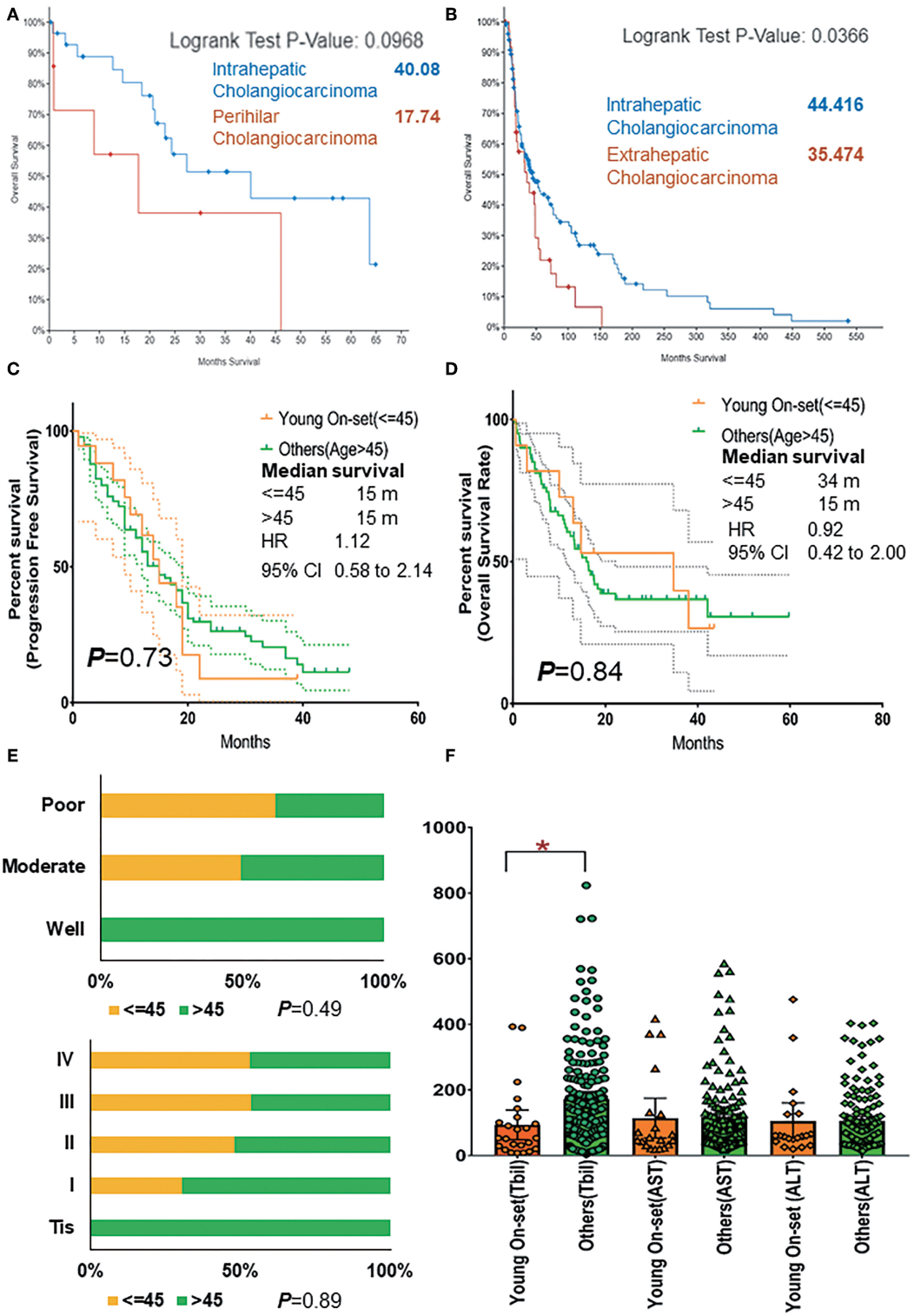


FIGURE 4 | (A) Comparison of overall survival rate of patients with intrahepatic and perihilar CCA basing on cohorts 2 and 3. **(B)** Comparison of overall survival rate of patients with intrahepatic and extrahepatic CCA basing on cohorts 2 and 3. **(C)** The progression-free survival rate of AYAs and others (age >45) with pCCA basing on cohort 1. **(D)** The overall survival rate of AYAs and others (age >45) with pCCA basing on cohort 1. **(E)** The proportion and ratio of different grades of differentiation and different pathological stages in AYA and others (age >45) group. **(F)** The comparison of TBil, AST, and ALT expression in AYA (<=45, young-onset) group and other (>45) group. ALT, alanine transaminase; AST, aspartate transaminase; AYA, adolescents and young adults; CCA, cholangiocarcinoma; pCCA, perihilar CCA; TBil, total bilirubin (blood). *$P < 0.05$.

of early-onset biliary cancers. Further studies including more samples are essential to investigate whether ASXL1 and KMT2C could be considered potentially targetable genomic signatures for young patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of the Renji Hospital and Study Group of Biliary Surgery of the surgical branch of the Chinese Medical Association. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HF and JW conceived the project, designed the study, and drafted the manuscript. HF and HT designed the study, wrote and revised the manuscript, and approved the final submission. HF, HT, and JW revised the manuscript and approved the final submission. JY, WC, and MH were involved in the design of the study. All authors read and approved the manuscript. All authors qualify as per ICJME criteria for authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01439/full#supplementary-material

Supplemental Figure 1 | (A) The mutation frequency of FGFR2, PBRM1, ERBB3 elevated in patients of AYA (<=45) group; The mutation frequency of BAP1, KRAS, SMAD4 elevated in patients of others (>45) groups basing on cohort 3 (MSKCC); **(B)** A summary of presentative mutation in AYA and other groups basing on cohort 3 (MSKCC). AYA, Adolescents, and young adults; mut, mutation. **(C)** The difference of RNA expression between the two groups basing on cohort 2.

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Meta-Analysis of Hematological Biomarkers as Reliable Indicators of Soft Tissue Sarcoma Prognosis

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Background: Several recent studies have reported the reliable prognostic effect of hematological biomarkers in various tumors. Yet, the prognostic value of these hematological markers in soft tissue sarcoma (STS) remains inconclusive. Thus, the aim of this meta-analysis was to check the effect of hematological markers on the prognosis of STS.

Methods: We systematically searched for relevant papers published before October 2019 in the PubMed and EMBASE databases. Overall survival (OS) and disease-specific survival (DSS) were the primary outcome, whereas disease-free survival was the secondary outcome. A thorough study of hazard ratios (HR) and 95% of confidence intervals (CIs) was done for determining the prognostic significance.

Results: We performed 23 studies that comprised of 4,480 patients with STS. The results revealed that higher neutrophil-to-lymphocyte ratio (NLR), C-reactive protein (CRP), and platelet-to-lymphocyte ratio (PLR) were associated with poor OS/DFS (HR = 2.08/1.72, for NLR; HR = 1.92/1.75, for CRP, and HR = 1.86/1.61, for PLR). In contrast, a low lymphocyte-to-monocyte ratio (LMR) was relate to worse OS/DFS (HR = 2.01/1.90, for LMR). Moreover, pooled analysis illustrated that elevated NLR and CRP represents poor DSS, with HRs of 1.46 and 2.06, respectively. In addition, combined analysis revealed that higher Glasgow prognostic score (GPS) was linked to an adverse OS/DSS (HR = 2.35/2.77).

Conclusion: Our meta-analysis suggested that hematological markers (NLR, CRP, PLR, LMR, and GPS) are one of the important prognostic indicators for patients affected by high-grade STS and patients with the STS being located in the extremity.

Keywords: soft tissue sarcoma, meta-analysis, hematological markers, prognosis, biomarker, inflammation

INTRODUCTION

Rationale

Soft tissue sarcoma (STS) is a relatively rare, heterogeneous tumor derived primarily from the mesodermal layer. Approximately 12,750 new cases and 5,270 deaths were reported in 2019 (1, 2). Several prognostic factors including tumor size, depth, histologic tumor grade, and patient age have proven effective in guiding the design of treatment regimens for STS (3). Nevertheless, mortality in

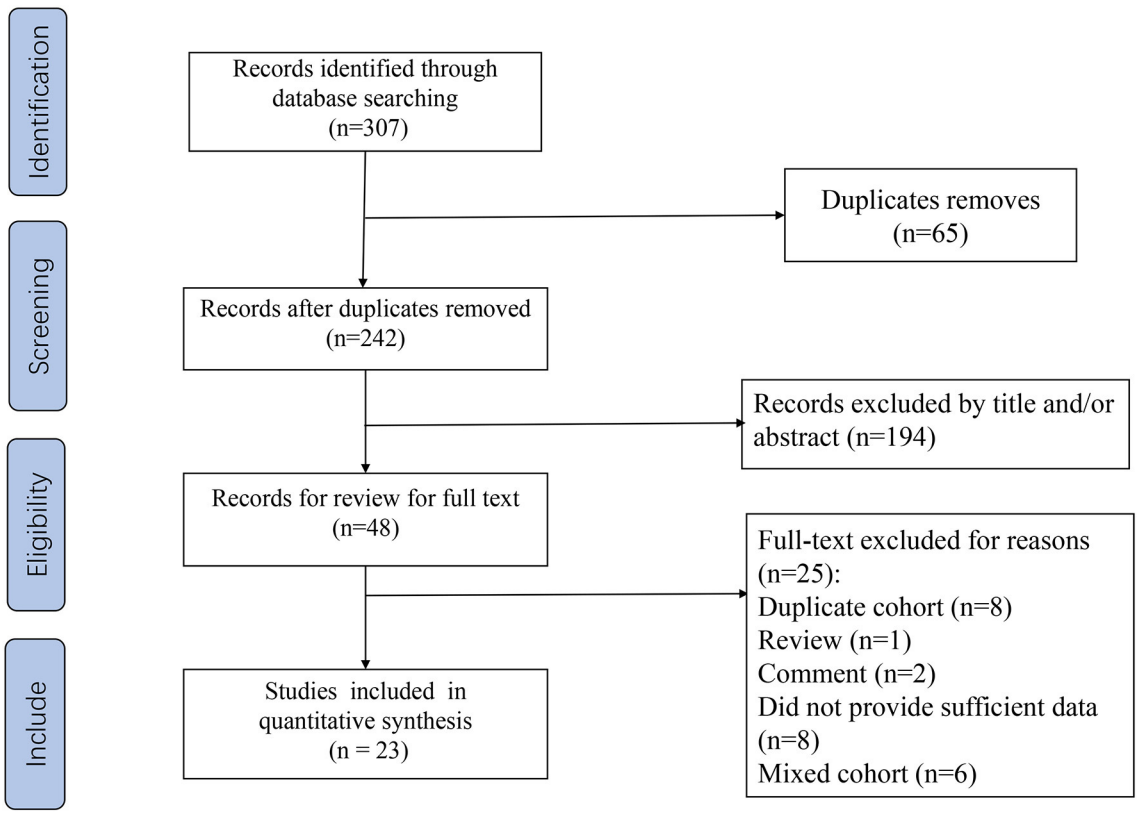

FIGURE 1 | Flow chart of the included studies.

patients with high-grade tumors is nearly 50%, primarily due to development of locally relapsed or metastatic tumors. Hence, more accurate predictive factors are required to allow for development of personalized treatment plans for high risk patients (4). Identifying accurate and novel biomarkers will provide improved treatment options and surveillance methods for STS.

For these novel biomarkers to provide more accurate diagnosis of patients with high risk of recurrence and metastasis, they must be readily accessible via non-invasive procedures and cost-effective. Accumulating evidence suggests that inflammatory cells and proteins play a key role in tumor development (5). Inflammation in the tumor microenvironment promotes angiogenesis, tumor invasion, and metastasis, subverts both the adaptive and innate immune responses while also increasing tumor cell proliferation and enhanced survival (5, 6). Fortunately, clinical routine tests, many of which are readily available and consist of inexpensive hematological markers, such as the NLR, CRP, PLR, LMR, and Glasgow prognostic score (GPS), can reflect the systemic inflammatory status. Notably, the aforementioned markers show reliable prognostic value for various tumors (7–13).

Objectives and Research Question

Inflammatory hematological biomarkers that have proven effective as prognostic factors in other tumors, may offer similar prognostic roles for STS. Although, several recent retrospective studies have demonstrated prognostic significance for some of these biomarkers in STS patients, the prognostic efficacy of several other markers have yet to be fully characterized. Therefore, the primary purpose of this meta-analysis was to explore the prognostic role of hematological biomarkers in STS.

METHODS

Search Strategies

Published reports before October 2019 and available in PubMed and EMBASE were retrieved through a systematic literature search. The keywords were as follows: hematologic markers, neutrophil-to-lymphocyte ratio (NLR), C-reactive protein (CRP), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), GPS, STS, prognosis, survival, and mortality. Since this is a meta-analysis and all data are collected from previously published studies, no ethical approval is required.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (1) diagnosis of STS based on pathological examination; (2) the study assessed the prognostic value for a minimum of one hematologic marker through overall survival (OS), disease-specific survival (DSS), and/or disease-free survival (DFS); (3) hazard ratio (HR) was

employed with a 95% confidence interval (CI) to represent the prognostic value of biomarkers; (4) studies published in English.

Studies were excluded if: (1) reviews, letters, comments, and case reports; (2) subjects include patients with osteogenic tumors; (3) studies did not follow standard treatment guidelines (4) overlapping or duplicate studies; (5) studies not in English.

Data Extraction and Quality Assessment

Two investigators (LL and ZB) independently selected these studies. Discrepancies were resolved by consensus, and the following information was extracted from each study: first author's name, publication year, country, number of patients, treatment method, tumor stage, cut-off value, and survival outcomes. HRs were primarily collected from multivariate analysis; in the case of no relevant data, univariate analysis was adopted. Two investigators used the Newcastle-Ottawa scale (NOS) to examine the quality of the reference articles. Studies with NOS scores ≥ 6 were included in our meta-analysis since they are considered as high-quality studies (14).

Data Analysis

Considering the similar survival outcomes, we combined DSS, sarcoma-specific survival (SSS), cancer-specific survival (CSS), and regarded them as DSS. In addition, recurrence-free survival (RFS), progression-free survival (PFS), and DFS were combined as DFS. The hematological biomarkers-survival outcome relationship was assessed by means of studying hazard ratio and 95% CI. The Cochrane *Q-test* and I^2 statistics were used to assess the heterogeneity among the studies. A random effects model (Der Simonian-Laird method) was employed in the case of any significant heterogeneity ($P < 0.05$ and $I^2 > 50\%$) (15), otherwise the fixed-effect model (Mantel-Haenszel method) was applied (16). In addition, subgroup analysis by treatment method, tumor stage, and ethnicity of NLR, CRP, and PLR was conducted. With the help of Stata software, version 12.0 (Stata corporation, College Station, TX, USA), publication bias was performed, whereas evaluation was completed by means of Begg's funnel plots, Egger's tests as well as the trim and fill method (17). Data analyses were conducted by RevMan5.3 (Cochrane Collaboration) and two-side $P < 0.05$ was considered to be statistically significant.

RESULTS

Study Selection and Characteristics

Our flow chart for data retrieval from publications is shown in **Figure 1**. The search strategy identified 307 potential records

TABLE 1 | Baseline characteristics of studies included in the meta-analysis.

References	Year	Country	Sample size	Treatment	Stage	Cut-off value	Makers	Outcome
Idowu et al. (18)	2012	UK	83	Surgery	Non-metastatic	5	NLR	OS RFS
Marshall et al. (19)	2017	Japan	75	Mixed	Mixed	NA	CRP	OS
Nakamura et al. (20)	2012	UK	312	Surgery	Non-metastatic	10	CRP	DSS RFS
Szkandera et al. (21)	2014	Austria	170T/170V*	Surgery	Non-metastatic	5/200/2.85	NLR/PLR/LMR	OS DFS CSS
Panotopoulos et al. (22)	2015	Austria	85	Surgery	Mixed	NA/8.7	NLR/CRP	OS DSS
Jiang et al. (23)	2015	China	142	Mixed	Metastatic	1	NLR	OS PFS
Nakamura et al. (24)	2017	Japan	47	Mixed	Metastatic	5,3,2	CRP	DSS
Chan et al. (25)	2018	Singapore	529L/183M†	Surgery/Mixed	Non/Metastatic	2.5/184/2.4	NLR/PLR/LMR	OS RFS
Park et al. (26)	2019	Korea	99	Surgery	Non-metastatic	1.95/1.4	NLR/CRP	OS DFS
Sasaki et al. (27)	2018	Japan	103	Mixed	Mixed	5/NA/1	NLR/PLR/GPS	OS
Liang et al. (28)	2017	China	206	Surgery	Mixed	1.64/151.9/1	NLR/PLR/GPS	OS DFS
Maretty-Kongstad et al. (29)	2017	Denmark	818/403‡	Mixed	Non-metastatic	NA/NA/1	NLR/CRP/GPS	DSS
Nakamura et al. (30)	2015	Japan	139	Surgery	Non-metastatic	1	GPS	DSS EFS
Szkandera et al. (31)	2013	Austria	304	Surgery	Mixed	6.9	CRP	OS DFS CSS
Choi et al. (32)	2014	Korea	162	Surgery	Non-metastatic	2.5/2	NLR/CRP	DSS
García-Ortega et al. (33)	2017	Mexico	169	Mixed	Mixed	3.5	NLR	OS
Chen et al. (34)	2019	China	42	Surgery	Mixed	2.73/103.89/4.2	NLR/PLR/LMR	OS DFS
Willegger et al. (35)	2017	Austria	132	Surgery	Mixed	8.7	CRP	OS SSS RFS
Tsuda et al. (36)	2017	Japan	202	Surgery	Non-metastatic	1	GPS	SSS EFS
Vasquez et al. (37)	2017	Peru	22	Mixed	Mixed	2/150	NLR/PLR	OS
Nakamura et al. (38)	2017	Japan	81	Surgery	Mixed	2.8/14	NLR/CRP	DSS
Nakamura et al. (39)	2012	Japan	102	Mixed	Non-metastatic	3	CRP	DFS
Cheng et al. (40)	2019	China	103	Mixed	Mixed	2.7/154.99/4.16	NLR/PLR/LMR	OS/PFS

NA, not available; OS, overall survival; DSS, disease-specific survival; SSS, sarcoma-specific survival; CSS, cancer-specific survival; DFS, disease-free survival; PFS, progression-free survival; RFS, recurrence-free survival.

**This study has validation set and training set, each set has 170 patients.*

† This study has non-metastatic and metastatic group.

‡ Four hundred and three patients have data on CRP.

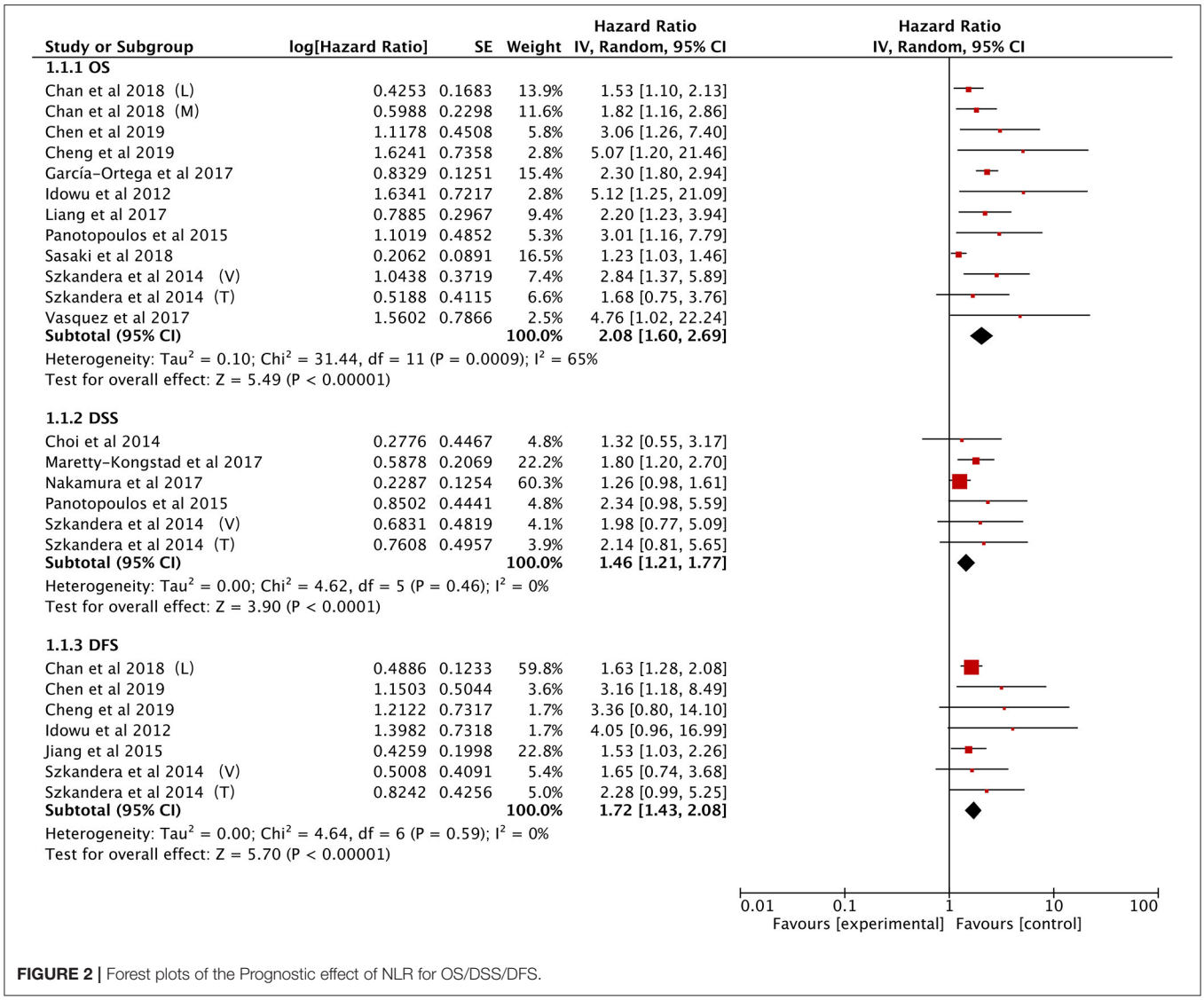

FIGURE 2 | Forest plots of the Prognostic effect of NLR for OS/DSS/DFS.

from the database. Ultimately, 23 studies involving 4,480 patients with STS met the inclusion criteria and were added into our meta-analysis. There were 15 studies for NLR, 11 for CRP, 7 for PLR, 4 for LMR, and 5 for GPS. The size of the samples ranged from 22 to 818. All studies collected data retrospectively. The mean NOS score was 6.95 and individual values ranged from 6 to 8. Further details of the studies are shown in **Table 1**.

Synthesized Findings

Correlation Between NLR and OS/DSS/DFS in STS

The data on prognostic value of NLR for OS were reported in 10 studies holding 1,964 STS patients (18, 21, 22, 25, 27, 28, 33, 34, 37, 40). Overall, elevated NLR was significantly associated with poor OS (HR: 2.08, 95% CI: 1.60–2.69, $P < 0.00001$), and due to the moderate heterogeneity observed, a random effect model was used ($I^2 = 65\%$; **Figure 2**). The NLR-OS correlation in synovial sarcoma and liposarcoma was shown in three studies and two studies, respectively (HR: 2.39, 95% CI: 1.89–3.02, $P <$ 0.00001 for synovial sarcoma; HR: 2.94, 95% CI: 1.81–4.77, $P < 0.0001$ for liposarcoma); no heterogeneity was detected ($I^2 = 0\%$; **Figure 3**). Only one study provided data on leiomyosarcoma, undifferentiated pleomorphic sarcoma, angiosarcoma, clear cell sarcoma, and rhabdomyosarcoma (HR: 1.62, 95% CI: 0.97–2.69, $P = 0.087$ for leiomyosarcoma; HR: 2.17, 95% CI: 1.49–3.16, $P = 0.0002$ for undifferentiated pleomorphic sarcoma; HR: 2.15, 95% CI: 1.29–3.59, $P = 0.0056$ for angiosarcoma; HR: 3.06, 95% CI: 1.26–7.40, $P = 0.013$ for clear cell sarcoma; HR: 4.76, 95% CI: 1.01–22.24, $P = 0.024$ for rhabdomyosarcoma).

The correlation between NLR and DSS was demonstrated in five studies comprising 1,486 STS patients (21, 22, 29, 32, 38). Collected data showed that poor prognosis of DSS was associated with high NLR (HR: 1.46, 95% CI: 1.21–1.77, $P < 0.0001$) without heterogeneity ($I^2 = 0\%$; **Figure 2**).

Six studies provided the data of NLR and DFS in STS (18, 21, 23, 25, 34, 40). The combined analysis indicated that NLR had a significant prognostic effect on DFS (HR: 1.72, 95% CI:

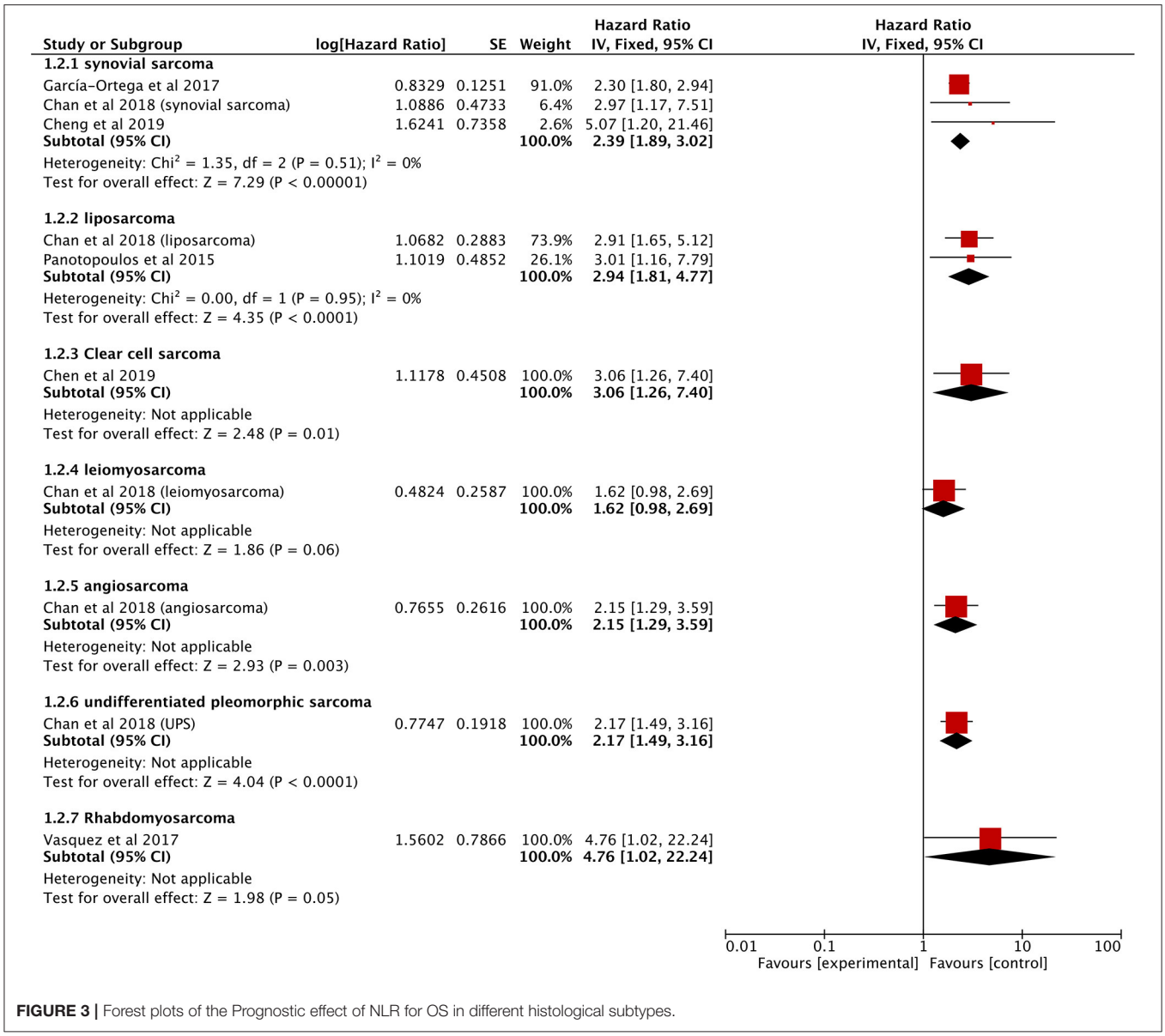

FIGURE 3 | Forest plots of the Prognostic effect of NLR for OS in different histological subtypes.

1.43–2.08, $P < 0.00001$), and no heterogeneity was detected ($I^2 = 0\%$; **Figure 2**).

Subgroup analysis illustrated that NLR was association with poor OS, DSS, and DFS in most subgroups, while the DSS Asia group had no significant prognostic value (**Table 2**).

Prognostic Value of Elevated CRP for OS/DSS/DFS

The effect of CRP on the STS prognosis was demonstrated in five studies (19, 22, 26, 31, 35). The analysis showed that a higher CRP level is a useful prognostic marker for predicting survival rate (HR: 1.92, 95% CI: 1.52–2.42, $P < 0.00001$) with no heterogeneity between studies ($I^2 = 0\%$; **Figure 4**). Seven studies reported the data on CRP and DSS (20, 22, 24, 29, 31, 32, 35). The random-effects model demonstrated that an elevated CRP levels had significantly prognostic value for DSS (HR: 2.06; 95% CI: 1.32–3.22; $P = 0.002$), but with significant heterogeneity ($I^2 = 84.0\%$; **Figure 4**). The correlation between CRP and DFS was demonstrated in five studies, and the pooled data illustrated that an elevated CRP level was associated with poor DFS (HR: 1.75; 95% CI: 1.38–2.23; $P < 0.00001$) (20, 26, 31, 35, 39). No heterogeneity ($I^2 = 0\%$; **Figure 4**) was observed. Subgroup analysis is shown in **Table 3**. The non-metastatic group did not show significant significance with regard to OS; the mixed treatment group and Asian ethnicity group did not show significant significance with respect to DSS.

Prognostic Effect of PLR for OS/DFS

The association between PLR and OS was demonstrated in seven studies (21, 25, 27, 28, 34, 37, 40). Elevated PLR was clearly

TABLE 2 | Subgroup analysis of the prognostic value of NLR.

Survival analysis	No. of studies	I^2 (%)	HR (95% CI)	*P*
OS				
Total	10	65%	2.08 (1.60–2.69)	$P < 0.00001$
Treatment				
Surgery	6*	14%	1.97 (1.56–2.48)	$P < 0.00001$
Mixed	5	82%	1.98 (1.27–3.08)	$P = 0.002$
Stage				
Non-metastatic	3†	33%	1.77 (1.34–2.33)	$P < 0.0001$
Metastatic	2	0%	2.06 (1.45–2.92)	$P < 0.0001$
Mixed	6	80%	2.33 (1.45–3.75)	$P = 0.0005$
Ethnicity				
Asian	5	59%	1.72 (1.29–2.31)	$P = 0.0003$
Latinos	2	0%	2.34 (1.84–2.98)	$P < 0.00001$
Caucasian	3	0%	2.60 (1.66–4.06)	$P < 0.0001$
DSS				
Total	5	0%	1.46 (1.21–1.77)	$P < 0.0001$
Treatment				
Surgery	4	0%	1.38 (1.11–1.71)	$P = 0.004$
Mixed	1	NA	1.80 (1.20–2.70)	$P = 0.004$
Stage				
Non-metastatic	3	0%	1.78 (1.29–2.46)	$P = 0.0005$
Mixed	2	45%	1.32 (1.04–1.67)	$P = 0.02$
Ethnicity				
Asian	2	0%	1.26 (1.00–1.60)	$P = 0.05$
Caucasian	3	0%	1.92 (1.39–2.66)	$P < 0.0001$
DFS				
Total	6	0%	1.72 (1.43–2.08)	$P < 0.00001$
Treatment				
Surgery	4	0%	1.76 (1.42–2.18)	$P < 0.00001$
Mixed	2	7%	1.62 (1.11–2.36)	$P = 0.01$
Stage				
Non-metastatic	3	0%	1.71 (1.37–2.13)	$P < 0.00001$
Metastatic	1	NA	1.53 (1.03–2.26)	$P = 0.03$
Mixed	2	0%	3.22 (1.43–7.27)	$P = 0.005$
Ethnicity				
Asian	4	0%	1.67 (1.37–2.04)	$P < 0.00001$
Caucasian	2	0%	2.14 (1.25–3.65)	$P = 0.005$

NA, not available.
**Chan 2018's study has both surgery cohort and mixed treatment cohort.*
† Chan 2018's study has both metastatic group and non-metastatic group.

associated with poor OS (HR: 1.86, 95% CI: 1.32–2.64, $P = 0.0004$), however, significant heterogeneity was observed ($I^2 = 85\%$; **Figure 5**).

The effect of PLR and DFS was reported in five studies (21, 25, 28, 34, 40). The fixed-effect model illustrated that an elevated PLR correlated with poor DFS (HR: 1.61, 95% CI: 1.32–1.95, $P < 0.00001$) with no heterogeneity among the studies ($I^2 = 0\%$; **Figure 5**).

Subgroup analytical studies illustrated that PLR had significant prognostic effect for OS and DFS in most subgroups, while the mixed treatment group on OS and DFS Caucasian ethnicity group had no significant prognostic value (**Table 4**).

Association Between LMR and OS/DFS in STS

A total of four studies provided LMR data on OS in STS patients (21, 25, 34, 40). The pooled data demonstrated that a low LMR had a visible prognostic effect on OS with an HR of 2.01 (95% CI: 1.65–2.45, $P < 0.00001$). No heterogeneity was observed ($I^2 = 0\%$; **Figure 6**).

The same four studies illustrated that LMR was also associated with DFS (21, 25, 34, 40). Alternatively, pooled data indicated that a low LMR had strong association with DFS (HR: 1.90, 95% CI: 1.49–2.43, $P < 0.00001$) and heterogeneity was not observed between studies ($I^2 = 0\%$; **Figure 6**).

Value of GPS for OS/DSS

Only two eligible studies explored the correlation between the GPS and OS (27, 28), and the combined data indicated that higher GPS scores correlated with much poorer OS (HR: 2.35; 95% CI: 1.64–3.36, $P < 0.00001$), without heterogeneity ($I^2 = 0\%$; **Figure 7**).

Three other studies show that high GPS is associated with poor DFS (29, 30, 36). The analysis showed that a higher GPS score is a useful prognostic marker for predicting DFS (HR: 2.77, 95% CI = 1.39–5.53, $P = 0.004$) with significant heterogeneity ($I^2 = 69\%$; **Figure 7**).

DISCUSSION

We performed a meta-analysis of 23 studies that were identified from multiple databases to examine the prognostic effect of hematological markers for STS. In our study, majority were high-grade and extremity tumors. The most common histological subtype was liposarcoma accounting for ∼830 cases, followed by malignant fibrous histiocytoma/undifferentiated pleomorphic sarcoma with ∼780 cases, and ∼550 cases of synovial sarcoma. The pooled data indicated that hematological markers, comprising NLR, CRP, PLR, LMR, and GPS, were associated with survival outcomes of STS; while high NLR, CRP, PLR, and GPS as well as low LMR were correlated with poorer prognosis. The results of the subgroup analysis also support our conclusions. Yet, many of the patients in our study were high grade patients with tumors located in the extremities, hence, these results should not be applied to all patients with STS. Patients with non-extremity and low-grade tumors require further analysis. Collectively, our findings suggest that these established markers, which can be tested using inexpensive, readily available assays, may serve as important biomarkers for the prognosis of high grade and extremity STSs.

Recently, the treatment for STS has changed allowing for improved overall prognosis. Despite some limitations, the clinical and pathological features have served as the primary prognostic factors for STS in recent decades. Innovative methodology has to must be applied to achieve improved early diagnosis of patients at risk of a specific outcome with acceptable cost (41). Molecular markers have shown reliable prognostic value in numerous types of cancer, some of which, including MDM2, MMP2, and P53, also exhibit a certain prognostic value in STSs. The MDM2 gene has been widely used in the diagnosis of STSs. A number of clinical trials targeting MDM2 gene drugs have recently

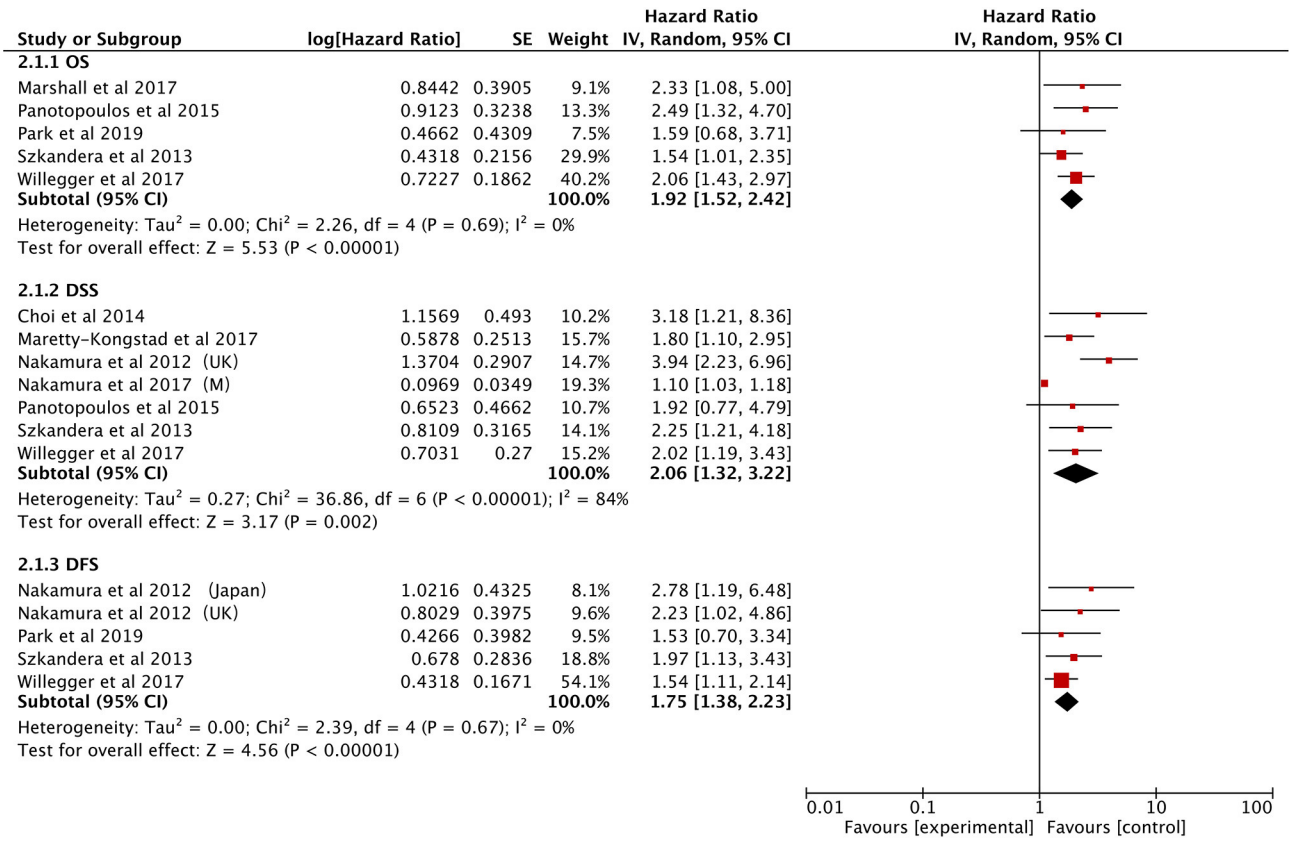

FIGURE 4 | Forest plots of the Prognostic effect of CRP for OS/DSS/DFS.

been conducted. Unfortunately, a meta-analysis shows that the MDM2 gene has a very limited role in prognosis (42, 43). Moreover, molecular detection technology must be improved to allow for reduced costs associated with evaluation (44, 45). Other markers, such as tumor necrosis, 18F-fluorodeoxyglucose positron emission tomography, and PD-1/PD-L1 have also demonstrated prognostic effects in STS. However, the clinical use of these markers is very limited (46, 47). Hence, none of these biomarkers are ready for clinical use.

In cancer patients, hematological markers serve as sensitive prognostic indicators, with inflammatory markers being the most reliable (7–13). The belief that a relationship exists between inflammation and tumor development can be traced back to the nineteenth century. As early as 1863, Rudolf Virchow observed leukocytes in tumor tissues and established this hypothesis. Due to the limitations of the times and technology, this speculation has been silent for many years. However, currently, our knowledge of inflammation in the tumor microenvironment has supported this hypothesis (48, 49). In fact, evidence now suggests that inflammation of the tumor microenvironment promotes tumorigenesis, growth, and metastasis, with a very prominent link between inflammation and tumors (5, 6, 49).

NLR is currently the most common hematological inflammation marker. Neutrophils can remodel the extracellular matrix and promote angiogenesis, which may stimulate tumor cell migration and metastasis. Furthermore, neutrophils significantly impact immunity by inhibiting cytolytic activity of lymphocytes, whereas tumor-infiltrating lymphocytes may restrict the metastatic outgrowth of cancer cells (50–52). In a previous study, Liu et al. (53) indicated that NLR may serve as a prognostic marker in both localized bone and STSs. However, osteoblastic tumors differ markedly from STSs in terms of treatment and prognosis. We, therefore, separated STS from osteogenic tumors and included a larger sample size.

The prognostic effect of CRP has been established in a variety of cancers. Tumor growth can lead to inflammation of tissues, thereby elevating the CRP level. Previous studies have preliminarily demonstrated the prognostic value of CRP in STS, however, there are certain limitations to these studies. For example, Li et al. (54) did not separate DSS from the OS even though these variable constitute two unique concepts by definition, especially when considering tumor prognosis. This can be observed from our conclusion. Compared to Xiaolin Wang's research (55), we have included more papers to provide a more comprehensive endpoint.

Previous studies have also shown that PLR exhibits reliable prognostic value in various tumors, such as those of ovarian cancer, pancreatic cancer, and bladder cancer. Platelets can mediate tumor cell growth, angiogenesis, and proliferation by

TABLE 3 | Subgroup analysis of the prognostic value of elevated CRP.

Survival analysis	No. of studies	I^2 (%)	HR (95% CI)	*P*
OS				
Total	5	0%	1.92 (1.52–2.42)	$P < 0.00001$
Treatment				
Surgery	4	0%	1.88 (1.48–2.40)	$P < 0.00001$
Mixed	1	NA	2.33 (1.08–5.00)	$P = 0.03$
Stage				
Non-metastatic	1	NA	1.59 (0.68–3.71)	$P = 0.28$
Metastatic	4	0%	1.95 (1.53–2.48)	$P < 0.00001$
Ethnicity				
Asian	2	0%	1.96 (1.11–3.46)	$P = 0.02$
Caucasian	3	0%	1.91 (1.48–2.46)	$P < 0.00001$
DSS				
Total	7	84%	2.06 (1.32–3.22)	$P = 0.001$
Treatment				
Surgery	5	0%	2.57 (1.91–3.45)	$P < 0.00001$
Mixed	2	73%	1.32 (0.83–2.10)	$P = 0.24$
Stage				
Non-metastatic	3	54%	2.72 (1.57–4.69)	$P = 0.0003$
Metastatic	1	NA	1.10 (1.03–1.18)	$P = 0.005$
Mixed	3	0%	2.08 (1.44–3.01)	$P < 0.0001$
Ethnicity				
Asian	2	77%	1.68 (0.62–4.54)	$P = 0.30$
Caucasian	5	16%	2.29 (1.76–2.97)	$P < 0.00001$
DFS				
Total	5	0%	1.75 (1.38–2.23)	$P < 0.00001$
Treatment				
Surgery	4	0%	1.68 (1.31–2.16)	$P < 0.0001$
Mixed	1	NA	2.78 (1.19–6.48)	$P = 0.02$
Stage				
Non-metastatic	3	0%	2.09 (1.31–3.31)	$P = 0.002$
Mixed	2	0%	1.64 (1.24–2.18)	$P = 0.0006$
Ethnicity				
Asian	2	2%	2.01 (1.13–3.57)	$P = 0.02$
Caucasian	3	0%	1.70 (1.30–2.22)	$P < 0.0001$

NA, not available.

TABLE 4 | Subgroup analysis of the prognostic value of PLR.

Survival analysis	No. of studies	I^2 (%)	HR (95% CI)	*P*
OS				
Total	7	85%	1.86 (1.32–2.64)	$P < 0.00001$
Treatment				
Surgery	4	0%	1.90 (1.53–2.35)	$P < 0.00001$
Mixed	4	84%	1.55 (0.93–2.58)	$P = 0.09$
Stage				
Non-metastatic	2	0%	1.76 (1.38–2.26)	$P < 0.00001$
Metastatic	1	NA	1.70 (1.28–2.26)	$P = 0.0002$
Mixed	5	80%	2.09 (1.08–4.04)	$P = 0.03$
Ethnicity				
Asian	5	88%	1.72 (1.17–2.52)	$P = 0.006$
Caucasian	1	0%*	1.97 (1.20–3.25)	$P = 0.008$
Latinos	1	NA	4.73 (1.01–22.17)	$P = 0.05$
DFS				
Total	5	0%	1.61 (1.32–1.95)	$P < 0.00001$
Stage				
Non-metastatic	2	40%	1.56 (1.24–1.97)	$P = 0.0002$
Mixed	3	0%	1.71 (1.19–2.44)	$P = 0.003$
Ethnicity				
Asian	4	0%	1.67 (1.36–2.06)	$P < 0.00001$
Caucasian	1	NA	1.01 (0.50–2.04)	$P = 0.98$

NA, not available.
**Szkandera 2014's study has validation set and training set, each set has 170 patients.*

releasing vascular endothelial growth factor, hepatocyte growth factor, basic fibroblast growth factor, angiopoietin-1 together with other angiogenesis and tumor growth factors. Furthermore, platelets have a defined role in protecting tumor cells from immune elimination and supporting tumor metastasis (56–58). In this meta-analysis, we observed that elevated PLR was clearly related with poor OS and DFS, consistent with the findings of previous studies. To our knowledge, this study is the first meta-analytical study that conducted research on the prognostic effect of PLR in STS patients.

Recent studies have also provided insights into the prognostic value of LMR. In fact, it has been suggested that LMR is a better prognostic indicator. Further, studies have highlighted the importance of tumor-associated macrophages. Hence, TMA derived from peripheral blood monocytes may support tumor progression and angiogenesis through secretion of growth factors and cytokines (59). This is also the first meta-analytical study, to our knowledge, to investigate LMR prognostic value in STS patients. However, only three studies were qualified for our analytical study, and subsequent studies are required.

There is also an increasing interest in scoring based on the inflammatory biomarkers. GPS is now used to predict various tumor prognoses (12). Glasgow's prognosis score consists of CRP and albumin as albumin levels in plasma reflect both the patient's nutritional level and systemic inflammation. However, most high scores are caused by abnormalities in CRP. Implying that the score is based on systemic inflammation. The significant correlation between GPS and STS is what our study demonstrated, with no similar meta-analysis previously performed.

Our study also has several limitations. First, we need to acknowledge that we cannot correct the histological subtype, a confounding factor that may affect outcomes. We have done our best to analyze histological subtypes. However, only three studies provided data on synovial sarcoma, two studies provided data on liposarcoma, and one provided data on clear cell sarcoma, angiosarcoma, undifferentiated pleomorphic sarcoma, rhabdomyosarcoma, and leiomyosarcoma. Results for a single subtype suggest that NLR has prognostic value in most subtypes, however, it is not possible to predict the prognosis of leiomyosarcoma. Thus, more research on specific subtypes is needed to further validate our results. Second, since some studies did not include multivariate analysis data, we included a portion of univariate analysis. Third, the same blood markers have different cut-off values. However,

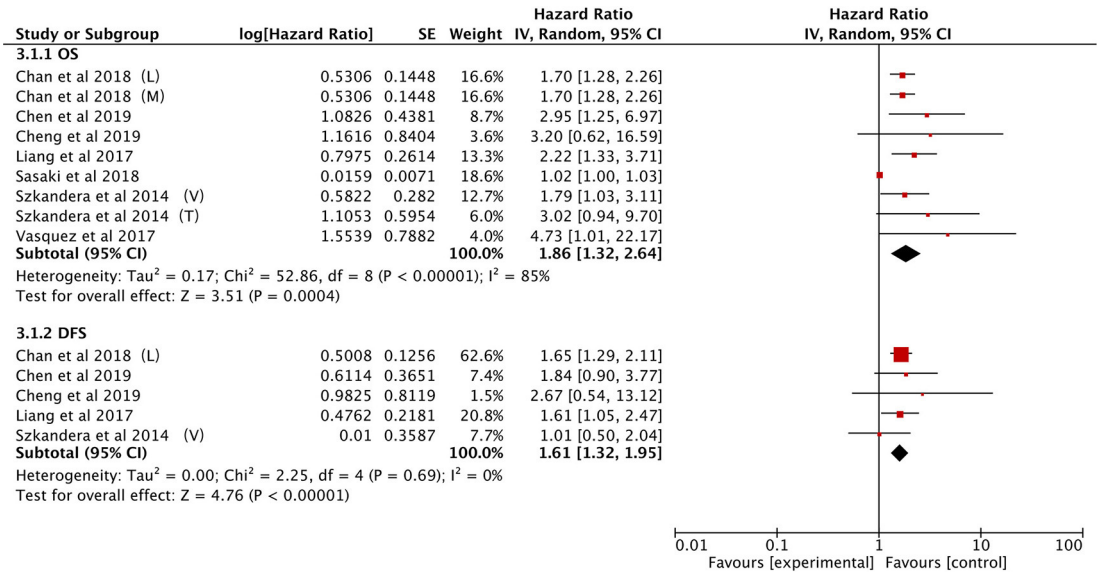

FIGURE 5 | Forest plots of the Prognostic effect of PLR for OS/DFS.

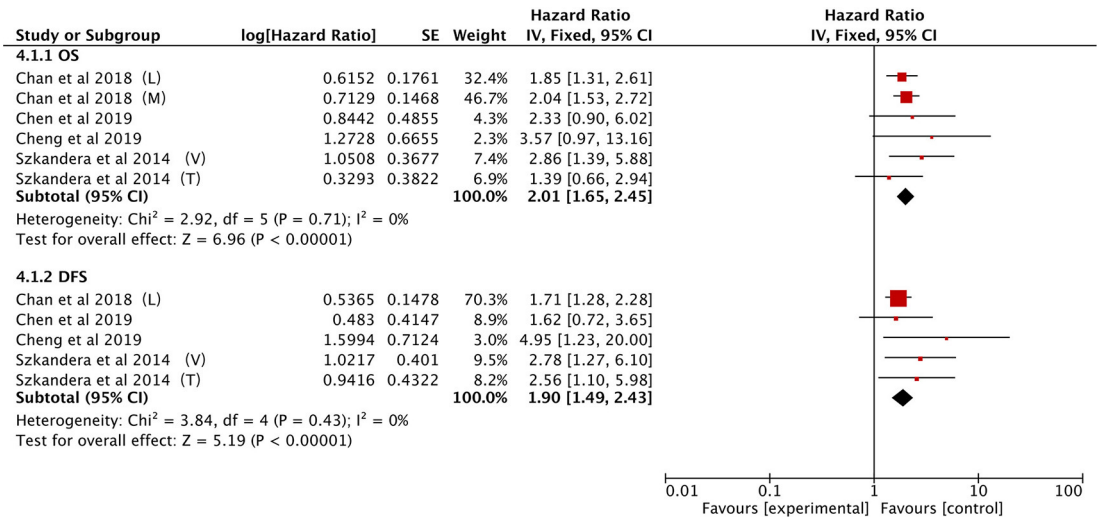

FIGURE 6 | Forest plots of the Prognostic effect of LMR for OS/DFS.

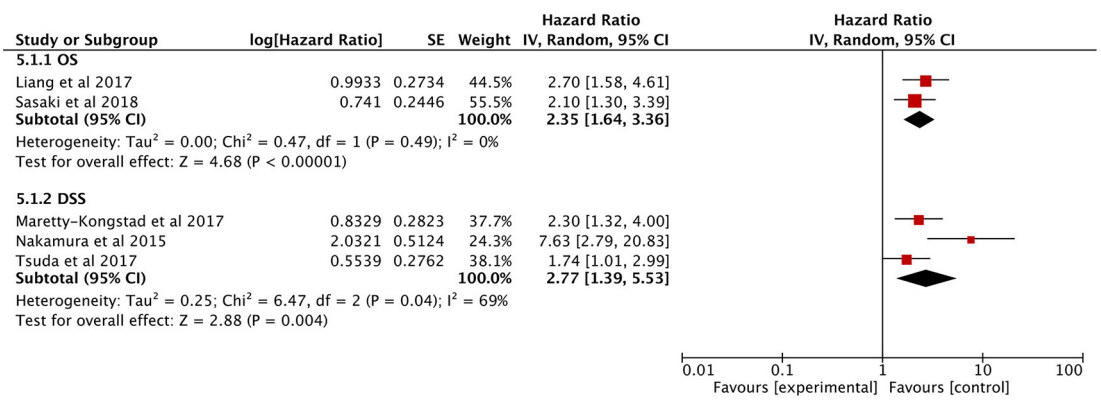

FIGURE 7 | Forest plots of the Prognostic effect of GPS for OS/DSS.

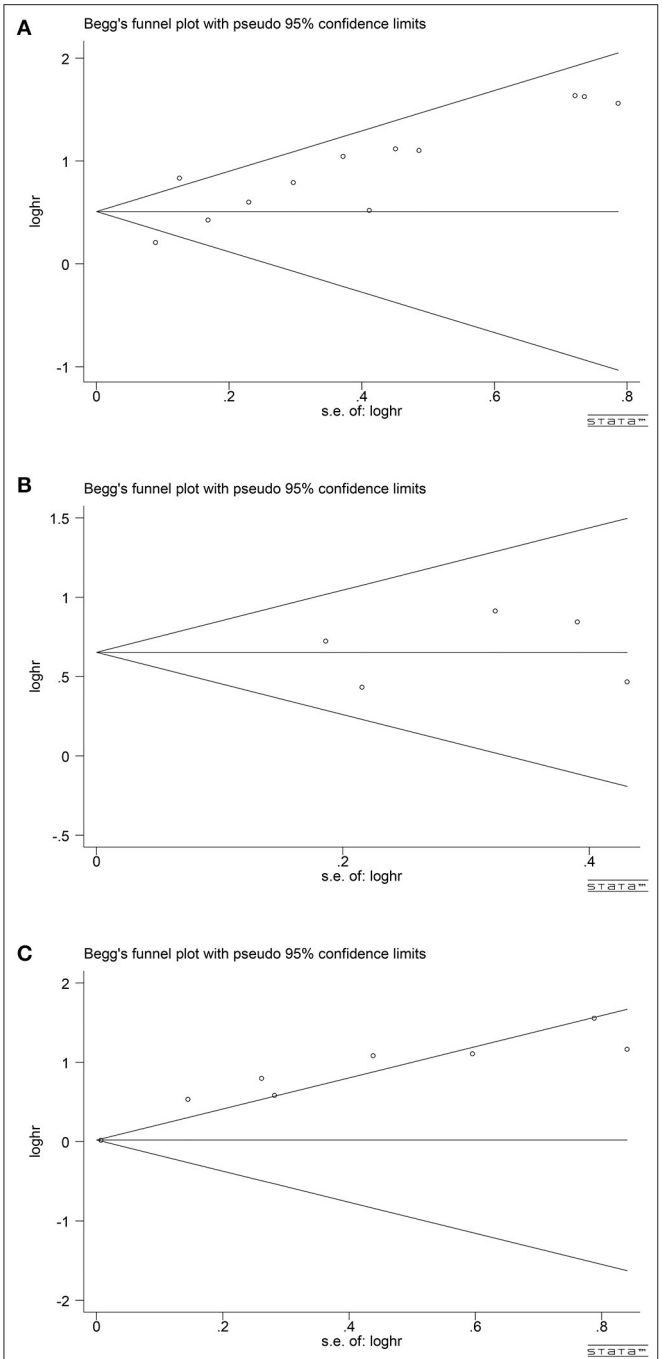

FIGURE 8 | Analyses of publication bias for the relationship between NLR/CRP/PLR and OS **(A)** Begger's funnel plot for NLR. **(B)** Begger's funnel plot for CRP. **(C)** Begger's funnel plot for PLR.

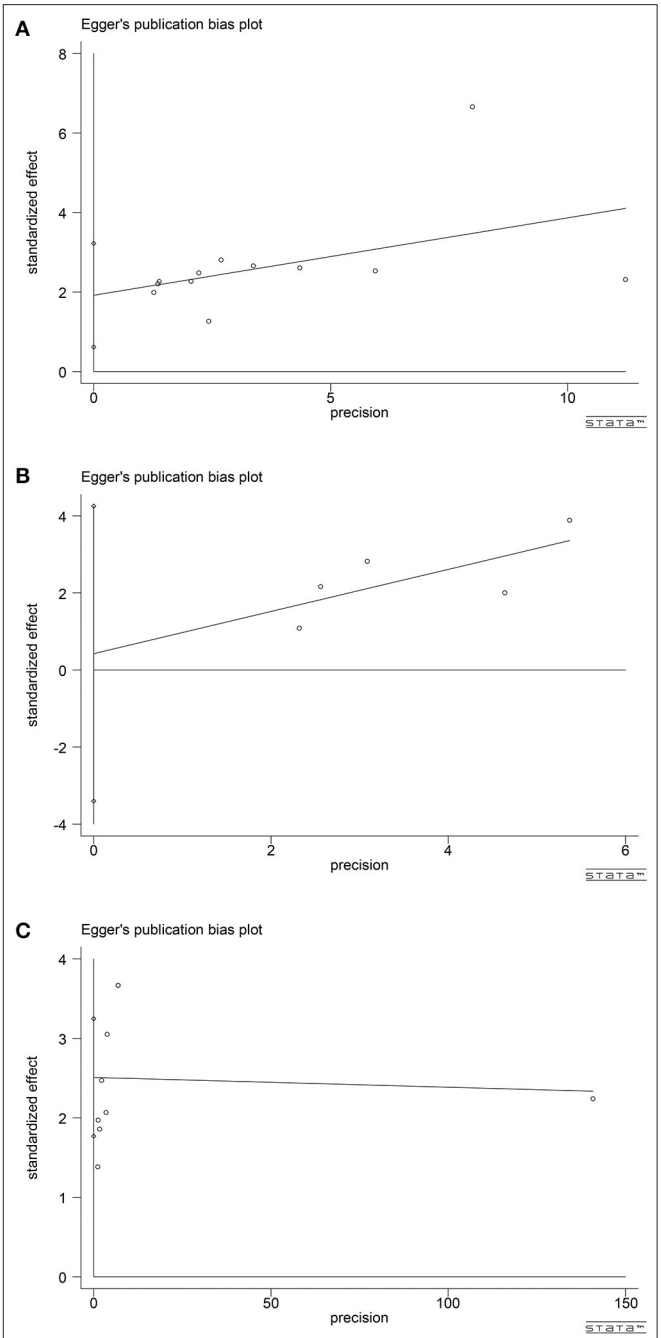

FIGURE 9 | Analyses of publication bias for the relationship between NLR/CRP/PLR and OS **(A)** Egger's publication bias plot for NLR. **(B)** Egger's publication bias plot for CRP. **(C)** Egger's publication bias plot for PLR.

since there have been no studies to compare the prognostic effects of different cutoff values, the optimal value cannot be evaluated. Nevertheless, our meta-analysis is the largest study to investigate the prognostic value of hematological markers in STSs. Compared to previous studies, we have included a larger sample size and excluded confounding factor of osteogenic tumors. Moreover, we are the first, to our knowledge, to investigate the prognostic value of multiple markers in STSs. These factors reinforce the strengths of our meta-analysis.

PUBLICATION BIAS

According to the publication-bias-plot shown in **Figures 8**, **9**, the bias was insignificant with regards to the prognostic value of NLR/CRP/PLR for OS. The Begg's p and Egger's p for OS were 0.115 and 0.008, respectively. Calculate new

HR using trim and fill methods (HR: 1.80; 95% CI: 1.42–2.28; $p < 0.001$; random effects). No publication bias was observed in the prognostic value of CRP for OS. The Begg's p and Egger's p for OS were 1.000 and 0.748. Among the seven included studies for PLR on OS, the Egger's test depicted proof of publication bias ($p = 0.000$), whereas the Begg's test did not ($p = 0.144$). Therefore, we used the trim and fill method allowing the new HRs to retain statistical significance (HR: 1.58; 95% CI: 1.17–2.13; $p < 0.001$; random effects).

CONCLUSIONS

Our research shows that hematological markers are one of the important prognostic indicators for patients affected by high-grade STS and patients with the STS being located in the extremity. Large-scale prospective studies are needed, especially studies targeting specific STS subtypes, to further validate our results.

AUTHOR CONTRIBUTIONS

L-QL collected and analyzed the data and wrote the paper. Z-HB and L-HZ assisted in collecting the data and participated in the writing. YaZ, X-CL, YiZ, JW, and Y-KL assisted in the design of this study. J-ZL was responsible for all the integrity of data and the accuracy of data analysis. All authors have thoroughly revised the manuscript.

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Cisplatin Resistance in Osteosarcoma: *In vitro* Validation of Candidate DNA Repair-Related Therapeutic Targets and Drugs for Tailored Treatments

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Treatment of high-grade osteosarcoma, the most common malignant tumor of bone, is largely based on administration of cisplatin and other DNA damaging drugs. Altered DNA repair mechanisms may thus significantly impact on either response or resistance to chemotherapy. In this study, by using a panel of human osteosarcoma cell lines, either sensitive or resistant to cisplatin, we assessed the value as candidate therapeutic targets of DNA repair-related factors belonging to the nucleotide excision repair (NER) or base excision repair (BER) pathways, as well as of a group of 18 kinases, which expression was higher in cisplatin-resistant variants compared to their parental cell lines and may be indirectly involved in DNA repair. The causal involvement of these factors in cisplatin resistance of human osteosarcoma cells was validated through gene silencing approaches and *in vitro* reversal of CDDP resistance. This approach highlighted a subgroup of genes, which value as promising candidate therapeutic targets was further confirmed by protein expression analyses. The *in vitro* activity of 15 inhibitor drugs against either these genes or their pathways was then analyzed, in order to identify the most active ones in terms of inherent activity and ability to overcome cisplatin resistance. NSC130813 (NERI02; F06) and triptolide, both targeting NER factors, proved to be the two most active agents, without evidence of cross-resistance with cisplatin. Combined *in vitro* treatments showed that NSC130813 and triptolide, when administered together with cisplatin, were able to improve its efficacy in both drug-sensitive and resistant osteosarcoma cells. This evidence may indicate an interesting therapeutic future option for treatment of osteosarcoma patients who present reduced responsiveness to cisplatin, even if possible effects of additive collateral toxicities must be carefully considered. Moreover, our study also showed that targeting protein kinases belonging to the mitogen-activated protein kinase (MAPK) or fibroblast growth factor receptor (FGFR) pathways might indicate new promising therapeutic perspectives in osteosarcoma, demanding for additional investigation.

Keywords: osteosarcoma, DNA repair, cisplatin, drug resistance, chemotherapy, targeted drugs, tailored treatment

INTRODUCTION

Osteosarcoma (OS) is the most common malignant tumor of bone, which accounts for about 5% of childhood and adolescence neoplasms. High-grade OS is usually treated with neoadjuvant chemotherapy protocols based on cisplatin (CDDP), doxorubicin, methotrexate, and ifosfamide. However, despite this aggressive approach, 35–45% of patients still recur and experience an unfavorable outcome (1–5).

Three out of the four conventional drugs, which are most commonly used in first-line chemotherapy for high-grade OS, induce DNA damages either directly (CDDP and ifosfamide) or indirectly (doxorubicin). Therefore, resistance mechanisms related to DNA damage response can significantly impact on OS chemotherapy unresponsiveness. Among these drugs, CDDP is the agent which has most extensively been studied in relation to DNA repair. A consistent body of evidence is showing that the onset of clinical unresponsiveness to CDDP usually creates further therapeutic complications, because patients can also become cross-resistant to the other DNA damaging chemotherapeutic drugs used in first- or rescue treatment protocols (4, 6).

One of the most important mechanisms of resistance against CDDP is repair of drug-induced DNA damages via different pathways, of which the most common is the nucleotide excision repair (NER) (4, 7, 8). We have recently obtained data indicating that protein overexpression of the NER gene *ERCC excision repair 1 (ERCC1)* negatively impacts on the clinical responsiveness to CDDP-based treatments and on patients' outcome (9). However, knowledge about the relevance of both *ERCC1* and other DNA repair genes for resistance to CDDP and DNA damaging drugs in OS still needs to be implemented.

In addition to NER, other DNA repair pathways, first of all the base excision repair (BER), have been indicated or proved to be implicated in CDDP resistance of several human tumors (10–12), but their relative impact significantly varies among different neoplasms and only very few information is available for OS (4).

Cellular response to CDDP-induced DNA damage is also mediated by downstream effects on cell cycle and mitosis regulation (7, 11). The interplay between DNA damage response and the proliferation machinery is based on the activity of several protein kinases, which in some tumors have been demonstrated to be involved in CDDP resistance (13). In human OS cells, we have obtained evidence of a possible involvement of aurora kinases in CDDP resistance (14) and of cyclin-dependent kinases (CDKs) in repair of CDDP-induced DNA damages (15), but this field of research still remains open.

Based on our previously (unpublished) gene expression analyses, we observed that CDDP-resistant human OS cell lines showed increased expression of several kinases in comparison with their corresponding parental cells. Among these kinases, 18 can be targeted by inhibitor drugs of which some have already entered clinical trials or have shown promising preclinical activities in human cancers different from OS.

In this study, we first confirmed the expression level of these 18 kinases in human OS CDDP-resistant variants in comparison with their parental cell lines.

Moreover, the role of genes belonging to NER or BER pathways and of the aforementioned 18 kinases for CDDP resistance in human OS cells was estimated, in order to indicate new candidate markers, which may be considered to overcome resistance to CDDP in OS patients.

Finally, the *in vitro* efficacy of drugs targeting the most significantly emerged genes or pathways has been assessed.

Abbreviations: ADD, additive; ANT, antagonistic; BER, base excision repair; CDDP, cisplatin; CDKs, cyclin-dependent kinases; CI, combination index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide; NER, nucleotide excision repair; OS, osteosarcoma; PDX, patient derived xenograft; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SYN, synergistic; TBST, Tris-Buffered Saline and Tween 20.

MATERIALS AND METHODS

Experimental Models

The *in vitro* studies were performed on the U-2OS and Saos-2 human OS cell lines and a panel of variants resistant to CDDP (U-2OS/CDDP300; U-2OS/CDDP1 μg; U-2OS/CDDP4 μg; Saos-2/CDDP300; Saos-2/CDDP1 μg; Saos-2/CDDP6 μg).

The U-2OS and Saos-2 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Variants resistant to CDDP were established by exposing the drug-sensitive U-2OS and Saos-2 parental cell lines to stepwise increasing concentrations of CDDP and characterized as previously described (16).

DNA fingerprint analyses of 17 polymorphic short tandem repeat sequences were performed for all cell lines, confirming their identity.

All cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with penicillin (20 U/ml)/streptomycin (20 U/ml) (Invitrogen Ltd., Paisley, UK) and 10% heat-inactivated fetal bovine serum (FBS; Biowhittaker Europe, Cambrex-Verviers, Belgium), and maintained at 37°C in a humidified 5% CO_2 atmosphere. Drug resistant variants were continuously cultured in presence of CDDP at the concentration used for their selection.

Gene Expression Analyses

Analyses focused on genes belonging to the NER and BER pathways, which are known to play key roles for CDDP resistance in several human cancers, and on the 18 druggable protein kinases selected on the basis of our previous observations, which indicated their increased expression in U-2OS- and/or Saos-2-derived CDDP-resistant variants in comparison with their parental cells (**Table 1**). Expression level of these genes was assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), in order to confirm their overexpression in CDDP-resistant variants compared to their parental cell lines. For each gene, 500 ng of total RNA were reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. cDNAs were aliquoted and stored at −20°C until use. To quantify the fold-change in gene expression, the TaqMan Gene Expression Assays listed in **Supplementary Table 1** were used

TABLE 1 | DNA repair and kinase genes analyzed in this study.

Pathway/family	Name (Gene ID)	Full gene name
Nucleotide excision repair (NER)	*ERCC1* (2067)	ERCC excision repair 1
	ERCC2/XPD (2068)	ERCC excision repair 2/Xeroderma pigmentosum D
	ERCC3/XPB (2071)	ERCC excision repair 3/Xeroderma pigmentosum B
	ERCC4/XPF (2072)	ERCC excision repair 4/Xeroderma pigmentosum F
	ERCC5/XPG (2073)	ERCC excision repair 5/Xeroderma pigmentosum G
	XPA (7507)	Xeroderma pigmentosum A
Base excision repair (BER)	*PARP1* (142)	poly(ADP-ribose) polymerase 1
	PARP2 (10038)	poly(ADP-ribose) polymerase 2
Kinases	*AKT3* (10000)	AKT serine/threonine kinase 3
	CDK3 (1018)	Cyclin dependent kinase 3
	CDK6 (1021)	Cyclin dependent kinase 6
	CDK8 (1024)	Cyclin dependent kinase 8
	CDK9 (1025)	Cyclin dependent kinase 9
	CDK10 (8558)	Cyclin dependent kinase 10
	FGFR1 (2260)	Fibroblast growth factor receptor 1
	FGFR2 (2263)	Fibroblast growth factor receptor 2
	FLT4 (2324)	Fms related tyrosine kinase 4
	MAP2K2 (5605)	Mitogen-activated protein kinase kinase 2
	MAP2K3 (5606)	Mitogen-activated protein kinase kinase 3
	MAP2K5 (5607)	Mitogen-activated protein kinase kinase 5
	MAP2K7 (5609)	Mitogen-activated protein kinase kinase 7
	MAPK1 (5594)	Mitogen-activated protein kinase 1
	MAPK3 (5595)	Mitogen-activated protein kinase 3
	PIK3C2A (5286)	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha
	PIK3C3 (5289)	Phosphatidylinositol 3-kinase catalytic subunit type 3
	PIK3CB (5291)	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta

on the ViiA 7 instrument (Applied Biosystems). GAPDH (Assay Hs99999905_m1; Applied Biosystem) was used as reference gene.

Gene Silencing

In a first set of experiments, each gene was silenced by transfecting cells with three different siRNAs specific for different regions of the same gene (customized Ambion Silencer Select siRNAs library, purchased from Thermo Fisher Scientific, Waltham, MA) for 24 h, whereas controls were cultured in presence of scrambled siRNAs. Transfection was performed by using each siRNA at a final concentration of 5 nM and 0.3 or 1.25 μl lipofectamin RNAiMAX (Invitrogen, Thermo Fisher Scientific) per well in a 96-well or 24-well plate according to the manufacturer's protocol. After 24 h, medium was changed and cells were maintained in siRNA-free medium for additional 48 h. The extent of gene silencing was estimated at 72 h for each siRNA by qRT-PCR on the ViiA 7 instrument (Thermo Fisher Scientific) in order to identify the siRNA with the strongest effect on mRNA down-regulation. Gene expression analysis was performed using the TaqMan® Gene Expression Cells-to-CT™ Kit (Invitrogen, Thermo Fisher Scientific) and appropriate TaqMan® Gene Expression Assays (Applied Biosystems) listed in **Supplementary Table 1**. GAPDH (Assay ID:Hs99999905_m1; Applied Biosystem) was used as reference gene.

The siRNAs producing the highest mRNA down-regulation were then selected to verify whether the inhibition of a specific gene expression was related to a corresponding increase in CDDP sensitivity. For this second set of experiments, 48 h after seeding and transfection, the cells were incubated with different dosages of CDDP for additional 48 h. Controls were incubated with scrambled siRNAs. The *in vitro* sensitivity to CDDP was estimated on the basis of drug dosage response curves, assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay kit (TACS MTT Cell Proliferation Assay, Trevigen, Gaithersburg, MD). For all cell lines, the IC50 value (CDDP concentration inducing 50% growth inhibition) was determined inside each experimental condition. To quantify the extent of the increased CDDP sensitivity after gene knock-down, ratios between the IC50 values of cells incubated with scrambled siRNAs and those of silenced cells were calculated.

Western Blot

Cells were cultured in petri dishes until confluence, harvested by scraping and lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific) and Benzonase (Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined by the Bradford Protein Assay (Bio-Rad Laboratories Italia, Segrate, Italy). Equal amounts of cell lysates (80 μg) were separated by SDS-PAGE on 4–20% gradient gels (Thermo Fisher Scientific) and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories Italia, Segrate, Italy). Then, membranes were blocked in 5% BSA in 1 X TBS containing 0.1% Tween-20 (TBST; Tris-Buffered Saline and Tween 20) and incubated in primary antibodies (**Supplementary Table 2**) overnight at 4°C, washed in 1 X TBST and incubated with the appropriate secondary antibody (goat anti-mouse or anti-rabbit IgG-HRP, Santa Cruz Biotechnology, 1:10,000) for 1 h. Blots were washed three times with 1 X TBST, detected with the SuperSignal West Pico Reagent (Thermo Fisher Scientific, Waltham, MA), and visualized in a ChemiDoc digital imaging station (Bio-Rad).

Protein loading was assessed by coomassie R-250 staining (Bio-Rad). Fold changes in protein expression level were determined by densitometric analysis of western blots and autoradiographs using the publicly available ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Drugs

The drugs targeting the prioritized genes/pathways, which have been tested for their *in vitro* efficacy, are listed in the **Supplementary Table 3**. Drugs were selected on the basis of their reported promising activity in other experimental models and/or their use in clinical trials for human tumors. NSC130813 (NERI02; F06), X80 and hypothemycin were purchased from Sigma-Aldrich-Merck KGaA (Darmstadt, Germany), whereas all the other drugs were purchased from Selleckchem Europe (Munich, Germany).

In vitro Drug Analyses

In vitro drug efficacy was assessed in terms of *in vitro* growth inhibition activity estimated with the MTT assay (as described above) on the two parental cell lines (U-2OS and Saos-2) and their CDDP-resistant variants with, respectively, the lowest and the highest resistance level (U-2OS/CDDP300, U-2OS/CDDP4 μg, Saos-2/CDDP300, and Saos-2/CDDP6 μg). For three drugs, X80, quercetin and SSR128129E, the CellTiter-FluorTM Cell Viability assay (Promega, Madison, WI) was used according to the manufacturer's protocol. For each cell line, the drug IC50 value was determined, in order to highlight the possible presence of cross-resistance due to the mechanisms present in CDDP-resistant variants.

The two most active drugs which emerged from these analyses were prioritized for further evaluations. The efficacy of the *in vitro* association of each prioritized drug with CDDP was determined after 96 h of combined treatment with the IC50 dosage of each drug. In drug sequence experiments, cell lines were sequentially exposed for 48 h to their corresponding IC50 dosage of CDDP and then to the IC50 dosage of each prioritized drug for additional 48 h. These combinations were then repeated with the opposite sequence. The type of interaction in terms of synergism, antagonism or additivity, was defined on the basis of the combination index (CI) of each two-drugs combination, which was calculated with the equation of Chou-Talalay by using the CalcuSyn software (Biosoft, Stapleford, UK). By following the CalcuSyn software indications, the drug–drug interaction was classified as synergistic (SYN) when CI was lower than 0.90, additive (ADD) when CI ranged between 0.90 and 1.10, or antagonistic (ANT) when CI was higher than 1.10.

RESULTS

Gene Expression Level

Expression levels of genes listed in **Table 1** were assessed by RT-PCR in U-2OS/CDDP-resistant variants and compared with those of their parental cell lines. As shown in **Table 2**, by considering a cut-off of at least 2.0-fold increase compared to parental cells, expression of all NER or BER genes was generally enhanced in CDDP300 and CDDP4μg resistant variants, with other level increases (1.8–1.9 fold) very closed to this cut-off value. Among kinases, those which showed evidence of a higher expression in at least two CDDP resistant variants included *CDK3, FLT4, MAP2K3, MAP2K5, MAPK1, MAPK3, PIK3C3*.

TABLE 2 | Expression of NER, BER, and kinase genes considered in this study assessed by RT-PCR.

Gene	fold vs. U-2OS		
	U2CDDP300	U2CDDP1ug	U2CDDP4ug
ERCC1	2.1	1.1	3.2
ERCC2	2.6	1.6	2.8
ERCC3	2.3	1.6	1.8
ERCC4	2.5	0.6	2.9
ERCC5	1.9	0.7	3.9
XPA	1.9	0.4	1.8
PARP1	2.7	0.8	1.6
PARP2	3.2	0.9	1.6
AKT3	0.9	1.0	0.5
CDK3	3.3	2.2	3.3
CDK6	1.2	1.0	0.8
CDK8	1.3	1.5	1.8
CDK9	1.6	1.5	2.8
CDK10	1.3	1.5	1.1
FGFR1	1.4	1.5	1.0
FGFR2	1.2	0.3	0.2
FLT4	5.8	3.9	4.2
MAP2K2	1.9	1.8	2.0
MAP2K3	1.2	3.4	2.2
MAP2K5	1.8	2.6	6.1
MAP2K7	0.3	1.8	1.1
MAPK1	3.4	2.9	3.5
MAPK3	2.1	3.0	3.0
PIK3C2A	1.5	0.8	2.4
PIK3C3	3.6	2.6	4.5
PIK3CB	1.3	1.5	1.8

Table shows the fold-changes in the U-2OS/CDDP-resistant variants referred to its parental cell line. Highlighted values indicate fold-increases ≥ 2.0.

Screening and Selection of the Most Active siRNAs

RNA interference was used to determine the causal involvement in CDDP resistance of the genes listed in **Table 1** by silencing each gene in the U-2OS parental cell line and its CDDP-resistant variants (U-2OS/CDDP300; U-2OS/CDDP1 μg; U-2OS/CDDP4 μg). The most effective siRNAs were identified through an extensive RNA interference approach, in which each gene was silenced by using three different siRNAs. All the selected siRNAs (**Supplementary Table 4**) proved to efficiently down-regulate the expression of their target genes and were used for the next phases of the study.

Reversal of CDDP Resistance After Gene Silencing

Cell lines silenced with the siRNAs listed in the **Supplementary Table 4** and their related controls were treated with CDDP, in order to verify whether a specific gene down-regulation was associated with a corresponding increase of the

TABLE 3 | Fold-changes in cisplatin IC50 after gene silencing.

Gene	U-2OS	U-2OS/CDDP300	U-2OS/CDDP1 μg	U-2OS/CDDP4 μg
ERCC1	3.1	9.3	6.5	1.6
ERCC2/XPD	2.8	6.9	5.0	1.5
ERCC3/XPB	1.2	2.0	2.6	0.9
ERCC4/XPF	2.0	2.9	2.8	1.1
ERCC5/XPG	0.6	0.5	0.8	0.3
XPA	1.4	3.3	3.5	1.5
PARP1	0.3	0.6	1.0	0.7
PARP2	0.9	1.3	1.1	0.9
AKT3	1.6	1.3	0.9	1.1
CDK3	0.9	1.2	1.2	1.0
CDK6	0.8	0.9	0.9	1.4
CDK8	1.3	0.5	0.6	1.6
CDK9	1.2	0.6	1.0	1.2
CDK10	1.4	1.3	1.3	1.6
FGFR1	2.0	1.2	2.2	1.2
FGFR2	1.2	1.2	0.9	1.0
FLT4	0.8	1.0	0.9	0.8
MAP2K2	0.9	1.0	1.2	1.5
MAP2K3	0.6	1.0	1.4	2.1
MAP2K5	1.1	1.0	1.2	1.6
MAP2K7	0.9	1.0	1.3	1.5
MAPK1	0.6	0.8	1.2	1.1
MAPK3	1.3	1.0	1.3	2.6
PIK3C2A	0.6	0.8	1.1	1.8
PIK3C3	1.4	1.2	1.1	1.7
PIK3CB	0.8	1.4	1.2	2.3

Values indicate ratios between the cisplatin IC50 values of silenced cells and those of cells incubated with scrambled siRNAs (controls). Highlighted values indicate ratios ≥ 2.0.

in vitro CDDP sensitivity. **Table 3** shows the fold-changes in CDDP IC50 after gene silencing. As specified in the Materials and Methods section, these values represent the ratio between the CDDP-IC50 between cells incubated with scrambled siRNAs (controls) and those of silenced cells and, therefore, they reflect the increased sensitivity to CDDP consequent to each gene knock-down. By considering ratios > 2.0, results can be summarized as follows:

(i) Silencing of *ERCC1, ERCC2/XPD, ERCC3/XPB, ERCC4/XPF,* and *XPA* increased CDDP sensitivity in the U-2OS/CDDP300 and U-2OS/CDDP1 μg resistant variants. Silencing of *ERCC1, ERCC2/XPD,* and *ERCC4/XPF* increased CDDP sensitivity also in U-2OS parental cells
(ii) *FGFR1* silencing increased CDDP sensitivity in the U-2OS/CDDP1 μg and in parental cells
(iii) Silencing of *MAP2K3, MAPK3,* and *PIK3CB* was associated with an increase of CDDP sensitivity in the U-2OS/CDDP4 μg resistant variant.

Additional reversal activity of CDDP resistance, with IC50 ratios close to the 2.0 cut-off value, was observed for the knock-down of these and other genes (**Table 3**), even if this evidence was not taken into account for the candidate drug targets prioritization.

Candidate Drug Targets Prioritization

By coupling the results derived from the assessment of gene expression level in association with CDDP resistance (**Table 2**) and the evaluation of increase in CDDP sensitivity after gene silencing (**Table 3**), the following genes were selected as candidate drug targets and were prioritized for the next phases of the study: *ERCC1, ERCC2/XPD, ERCC3/XPB, ERCC4/XPF, XPA, MAP2K3, MAPK3,* and *PIK3CB*. *FGFR1* was also selected based on its ability to reverse CDDP resistance also if its expression was found to be moderately higher than in parental cells. All these genes showed evidence of increased expression in CDDP resistant variants and their knock-down proved to be associated with an enhancement of CDDP sensitivity.

Western Blot

To further validate these genes as candidate therapeutic targets to overcome CDDP-resistance, their expression at protein level was assessed by western blot in both U-2OS and Saos-2 parental cell lines and all their CDDP-resistant variants. All proteins encoded by these prioritized genes proved to be expressed in all cell lines. In the U-2OS series (**Figure 1A**), there was evidence of a trend toward an increased protein level in CDDP resistant variants for ERCC2, and in some variants for ERCC1, ERCC4, MAP2K3, FGFR1, and PI3K beta. In the Saos-2 series (**Figure 1B**), a more clear evidence of increased protein levels in CDDP resistant variants was observed for all prioritized genes, excepting ERCC2 and ERCC3.

When considered together, these results further supported the indication of all these genes as candidate drug targets.

Efficacy of Drugs Against Selected Candidate Targets

The *in vitro* activity of drugs listed in **Supplementary Table 3** was assessed by estimating their IC50 on parental cell lines (U-2OS and Saos-2) and on their resistant variants with the lowest and the highest CDDP resistance level (U-2OS/CDDP300; U-2OS/CDDP4 μg; Saos-2/CDDP300; and Saos-2/CDDP6 μg, respectively).

As shown in **Figure 2**, several drugs showed IC50 lower than 5 μM in all cell lines. In both U-2OS and Saos-2 drug sensitive and CDDP-resistant cells, X80 and SSR128129E showed very high IC50 values, whereas TGX221, GSK2636771, quercetin, and (at a lower extent) GDC0994 showed IC50 values higher than 5 μM.

In U-2OS variants (**Figure 2A**), higher IC50 values in CDDP-resistant variants compared to parental cell lines (indicating possible cross-resistance) were observed for TGX221, AZD6482, FR180204, AZD4547 and, at a lower extent, for quercetin. In Saos-2 variants (**Figure 2B**), a possible cross-resistance with CDDP was observed for GDC0994.

By considering together the findings obtained in both U-2OS and Saos-2 cell line series, the most active DNA repair-targeting agents, without evidence of cross-resistance, proved to

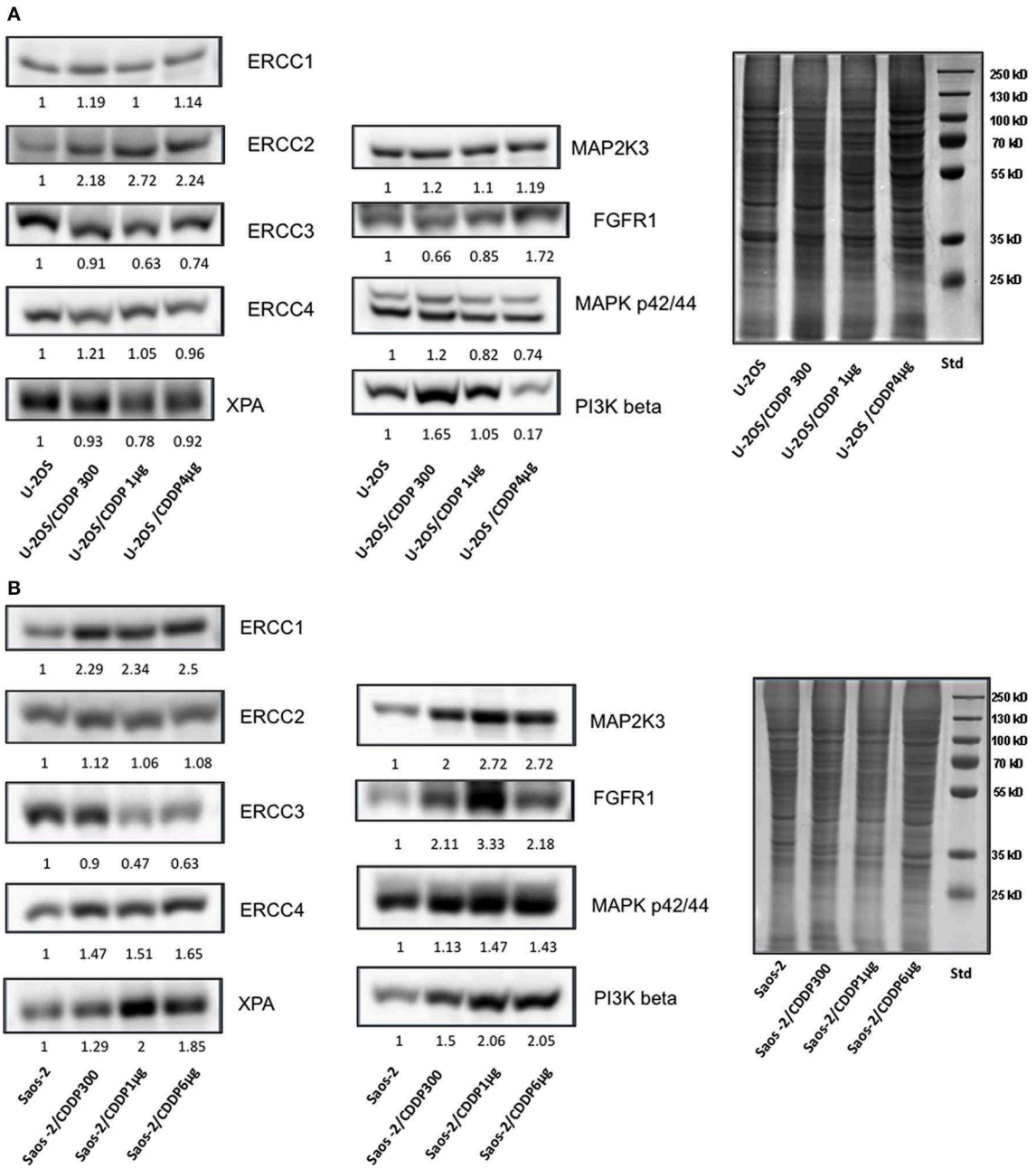

FIGURE 1 | Assessment by western blot of protein expression level in CDDP-resistant variants derived from U-2OS **(A)** and Saos-2 **(B)** in relation to their corresponding parental cells.

be NSC130813 (NERI02; F06; targeting the interaction between ERCC1 and ERCC4/XPF) and triptolide (targeting ERCC3/XPB).

The most active kinase-targeting drugs, without evidence of cross-resistance with CDDP, were ulixertinib (targeting the downstream MAPKs signaling pathway), hypotemycin (targeting the MAP2K pathway), PD173074 and FIIN-2 (both targeting FGFR1).

In order to verify whether treatment with each inhibitor drug was able to increase sensitivity to CDDP, the same group of cell lines were incubated with increasing CDDP concentrations in the absence (control) or presence of the IC20 dosage of each inhibitor drug. A ratio $\geq$ 2.0 (meaning a decrease of at least 2-fold of CDDP-IC50 in presence of the inhibitor drug) was considered as indication of a drug-induced CDDP sensitization.

NSC130813 (NERI02; F06) and triptolide proved to be the two drugs with the most relevant activity, being able to increase CDDP sensitivity for more than 2-fold in all the U-2OS and Saos-2 cell lines (**Table 4**).

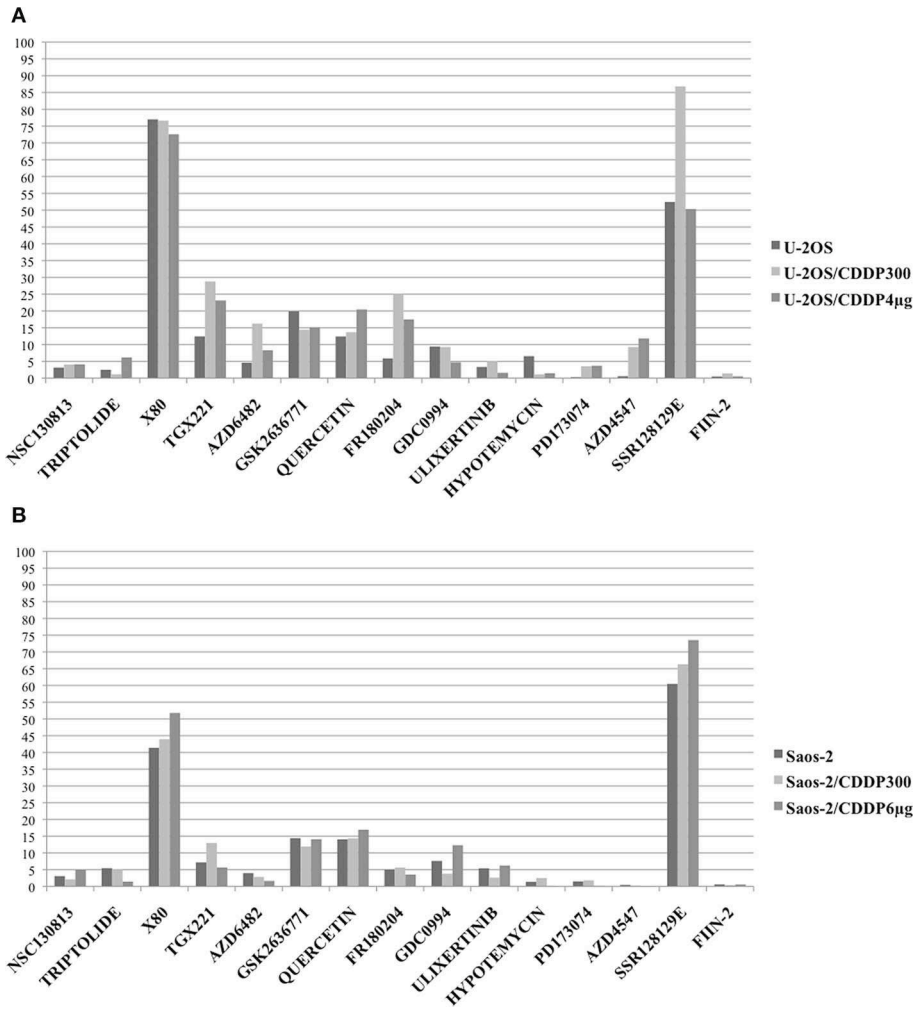

FIGURE 2 | *In vitro* activity of drugs against selected target genes was assessed by estimating their IC50 on parental cell lines U-2OS **(A)** and Saos-2 **(B)** and on their CDDP resistant variants with the lowest and the highest resistance level (U-2OS/CDDP300; U-2OS/CDDP4 μg; Saos-2/CDDP300; and Saos-2/CDDP6 μg). Graphs show the IC50 values (μM) determined after 96 h of *in vitro* drug treatment (Y axis). For triptolide, IC50 values are expressed in nM.

For all these reasons, NSC130813 (NERI02; F06) and triptolide were prioritized for evaluation in combination experiments with CDDP.

Combined Treatments

NSC130813 (NERI02; F06) and triptolide were tested in combination with CDDP, in order to verify whether these treatments lead to positive interactions. As shown in **Table 5**, association with CDDP produced positive (additive or synergistic) effects in both CDDP-sensitive and resistant cell lines. The only antagonistic interaction was observed in the U-2OS cell line treated with CDDP in association with NSC130813 (NERI02; F06).

Sequential drug exposure experiments (**Table 6**) mainly revealed antagonistic effects when CDDP was combined with triptolide, independently from the sequence of drug administration. Treatment with CDDP followed by NSC130813 (NERI02; F06) invariably produced antagonistic effects, whereas the opposite sequence proved to be mainly additive or synergistic (**Table 6**).

DISCUSSION

Many chemotherapeutic drugs, including several agents used in first-line and rescue chemotherapy protocols for OS exert their activity by directly or indirectly damaging DNA. Consequently, the ability of tumor cells to repair drug-induced DNA damages significantly impacts upon efficacy of these compounds (7, 8, 10, 11). Accordingly, the expression and activity of factors belonging to DNA repair pathways have been demonstrated to be involved in chemotherapy response and patients' outcome in different human tumors (7, 8, 12), with few findings also reported for OS (4, 9). This body of evidence has also indicated components related to DNA repair pathways as promising targets

TABLE 4 | Fold-decrease in cisplatin (CDDP) IC50 induced by targeted drugs.

Drug	U-2OS	U-2OS CDDP300	U-2OS CDDP4μg	Saos-2	Saos-2 CDDP300	Saos-2 CDDP6μg
NSC130813 (NERI02, F06)	12.6	2.1	2.0	16.3	13.1	3.8
Triptolide	2.0	3.7	2.3	3.7	4.2	2.4
X80	1.78	0.8	1.2	0.9	0.9	0.8
AZD6482	1.5	1.2	1.1	2.9	2.5	1.2
GSK2636771	1.2	1.5	0.8	5.0	4.5	1.3
Quercetin	1.0	1.3	0.8	2.8	1.2	1.4
TGX221	1.5	1.2	0.8	3.0	6.9	1.3
FR180204	1.5	1.2	1.1	2.5	5.2	1.4
GDC0994	1.6	2.0	1.1	2.5	2.9	0.9
Ulixertinib (BVD-523)	1.4	1.0	1.1	1.6	0.8	1.2
Hypothemycin	0.8	0.9	0.9	2.1	0.7	1.2
AZD4547	1.2	1.2	1.0	1.2	0.7	1.2
FIIN-2	2.4	1.4	0.8	1.7	1.3	2.5
PD173074	2.1	1.2	0.8	2.5	4.1	1.2
SSR128129E	1.2	0.6	1.8	0.9	0.7	1.8

Values indicate ratios between the CDDP IC50 of cells incubated with increasing CDDP concentrations in absence (reference) or presence of the IC20 dosage of each target inhibitor drug. Highlighted values indicate ratios ≥ 2.0. Data refer to the mean ratio value of three different experiments.

TABLE 5 | Interaction of NSC130813 and triptolide with cisplatin (CDDP) in drug association experiments.

Cell line	CDDP + Triptolide	CDDP + NSC130813
U-2OS	ADD (1.08)	ANT (1.36)
U-2OS/CDDP300	SYN (0.65)	ADD (0.97)
U-2OS/CDDP4 μg	SYN (0.59)	ADD (1.10)
Saos-2	SYN (0.41)	SYN (0.67)
Saos-2/CDDP300	SYN (0.45)	SYN (0.41)
Saos-2/CDDP6 μg	SYN (0.32)	SYN (0.71)

Legend: Data refer to at least two different determinations. Number in parenthesis indicate the combination index (CI) values. SYN, synergistic (CI < 0.90); ADD, additive (0.90 ≤ CI ≤ 1.10); ANT, antagonistic (CI > 1.10).

for innovative anticancer therapies, and several drugs interfering with these systems have entered phases I-II-III clinical trials (11, 17, 18).

Our study focused on a group of DNA repair genes and kinases, which we found to be upregulated in our panel of CDDP-resistant human OS cell lines, in order to verify whether they could be considered as new candidate therapeutic targets. In particular, analyses focused on genes belonging to the NER and BER pathways and on 18 druggable protein kinases, which resulted to be overexpressed in association with the development of CDDP resistance.

After a screening to identify the most effective siRNA to knock-down each prioritized gene, drug-sensitive and resistant cell lines were silenced and treated with CDDP, in order to identify those genes which down-regulation produced a corresponding increase of the *in vitro* CDDP sensitivity, confirming its involvement in reduced sensitivity to this drug. The genes that emerged to be most strictly related to CDDP unresponsiveness, were prioritized as candidate targets for the second phase of the study, in which their increased protein expression was confirmed in CDDP-resistant cells, justifying the subsequent *in vitro* studies of drugs interacting with these markers or their pathways.

The impact of these genes for DNA repair activity in our experimental models was further confirmed by functional analyses, in which the cells' capability to repair CDDP-induced DNA damages was assessed after silencing of each prioritized gene by the COMET assay (**Supplementary Material**). This evaluation showed that all these genes were, at different extent, significantly involved in this process since its knock-down produced a decrease of DNA repair activity in both sensitive and CDDP-resistant cell lines. These findings further support their value as candidate drug targets which may be considered for planning treatment strategies based on the synthetic lethality principle.

Drugs targeting the prioritized targets or pathways were selected on the basis of their reported promising activity in other experimental models and/or their use in clinical trials for human tumors. Among the 15 evaluated agents, TGX221, AZD6482, FR180204, AZD4547, GDC0994 and, at a lower extent, quercetin showed a reduced *in vitro* activity in CDDP-resistant variants compared to parental cell lines, suggesting the presence of cross-resistance mechanisms. The possible reasons for this apparent cross-resistance were not further explored because they were beyond the aims of this study. However, it can be hypothesized that cross-resistance might be due either to differential expression of transporters that recognize these drugs as substrate and efflux them out of the cells or to detoxification processes that are more active in CDDP resistant cells and inactivate these agents. Other reasons may be the activation of alternative or redundant pathways, which replace the function of the targeted pathway in CDDP resistant cells, which consequently become less sensitive to these drugs.

TABLE 6 | Interaction of NSC130813 and triptolide with cisplatin (CDDP) in drug sequence experiments.

Cell line	Treatment schedule			
	CDDP → triptolide	Triptolide → CDDP	CDDP → NSC130813	NSC130813 → CDDP
U-2OS	ANT (2.14)	ANT (1.38)	ANT (1.67)	ANT (2.29)
U-2OS/CDDP300	ANT (1.38)	ANT (5.02)	ANT (1.96)	SYN (0.73)
U-2OS/CDDP4 μg	ANT (4.55)	ANT (2.23)	ANT (5.20)	ADD (1.09)
Saos-2	ADD (1.08)	ADD (0.99)	ANT (2.45)	SYN (0.73)
Saos-2/CDDP300	ANT (2.32)	ANT (2.17)	ANT (2.45)	ANT (1.86)
Saos-2/CDDP6 μg	ANT (9.19)	ANT (1.85)	ANT (9.87)	SYN (0.88)

Legend: Data refer to at least two different determinations. Number in parenthesis indicate the combination index (CI) values. SYN, synergistic (CI < 0.90); ADD, additive (0.90 ≤ CI ≤ 1.10); ANT, antagonistic (CI > 1.10).

NSC130813 (NERI02; F06; targeting ERCC1 and ERCC4/XPF) and triptolide (targeting ERCC3/XPB) proved to be the two agents with the most relevant activity on both CDDP-sensitive and -resistant cell lines. Moreover, these two drugs did not show evidence of cross-resistance with CDDP and proved to reverse CDDP resistance in all drug-sensitive and -resistant cell lines. For these reasons, they were further tested in combined treatments (association and sequential exposure) with CDDP, in order to verify whether these combinations may lead to positive interactions.

When considered together, results obtained by the combined treatments indicated that NSC130813 (NERI02; F06) and triptolide have to be administered together with CDDP, in order to improve its efficacy in both drug-sensitive and resistant OS cells. If transferred to a clinical setting, this association has to be considered regarding possible effects of additive collateral toxicities.

NSC130813 (NERI02, F06), also known as [4-[(6-chloro-2-methoxy-9-acridinyl)amino]-2-[(4-methyl-1-piperazinyl)methyl]], is a compound which was shown to act synergistically with CDDP and mitomycin C by interfering DNA repair through the disruption of the interaction between ERCC1 and ERCC4/XPF (19). Targeting the ERCC1-ERCC4/XPF complex is an interesting approach to improve activity of DNA damaging drugs, because this complex plays a primary role in several DNA repair pathways, in addition to NER (19–21). The inhibition of ERCC1-ERCC4/XPF endonuclease activity is a relatively new strategy, which has been scarcely explored and for which no data have been reported yet for OS. Our study provided the proof-of-concept that targeting this complex may become an interesting future option also for OS treatment. Recent studies have provided important information that can be effectively used in the rational design of ERCC4/XPF inhibitors (10, 18, 20), which may therefore soon become available for clinical use.

Triptolide is a diterpene triepoxide isolated from a traditional Chinese medicinal plant with anti-inflammatory, immunosuppressive, contraceptive and antitumor activities (22). In the MG63 human OS cell line, triptolide proved to induce apoptosis and inhibit angiogenesis (23).

It has been demonstrated that triptolide covalently binds to human ERCC3/XPB, inhibiting its DNA repair-related activity (22, 24, 25). This ability to block DNA repair has important implications for the anticancer activity of CDDP, which effectiveness has been shown to be enhanced by the combined treatment with triptolide (26). In agreement with that, experimental studies confirmed that low concentrations of triptolide were able to potentiate the CDDP activity in human lung cancer (27) and human bladder CDDP-resistant cells (28).

On the basis of this body of evidence, we have explored whether in OS cells triptolide-mediated inhibition of NER may improve CDDP activity. Our findings indicated that inhibiting DNA repair through the simultaneous administration of CDDP and triptolide may be a new interesting treatment avenue to overcome CDDP resistance in OS.

In clinical setting, it is worthwhile noting that minnolide, a highly water-soluble analog of triptolide, has been recently included in trials for pancreatic cancer (ClinicalTrials.gov Identifier: NCT03117920), acute myeloid leukemia (ClinicalTrials.gov Identifier: NCT03347994), and different advanced solid tumors (ClinicalTrials.gov Identifier: NCT03129139), but results of these regimens are presently not available. Moreover, other triptolide derivatives and analogs have been used in clinical studies aimed to test their efficacy and safety (22).

Taken together these results indicated that targeting NER factors may have clinical relevance for OS treatment, with the hope that new drugs will become soon available, since few NER inhibitors have entered clinical trials so far.

In addition to DNA repair systems, different checkpoints may be induced by DNA damage to transiently delay or arrest cell cycle progression, providing time to the cell for repair before progressing into cell cycle or being addressed toward apoptosis (7, 11). Indeed, a variety of regulators including kinases, phosphatases, ubiquitin ligases, deubiquitinases, and

other protein modifying enzymes, have been shown to modulate the activity and levels of key proteins belonging to different DNA repair pathways (13). In particular, protein kinases have been indicated to be involved or interfere with response to drug-induced DNA damages (13), despite their actual role in this process must be carefully investigated and validated inside each specific tumor type and only few preliminary information has been reported for OS so far (14, 15).

In this study, we have determined the *in vitro* activity of 13 drugs inhibiting kinases pathways and proteins, which resulted to be overexpressed in U-2OS- and/or Saos-2-derived CDDP-resistant variants compared to parental cells. Among the tested kinase targeting drugs, GDC0994 (targeting the MAPK pathway) and PD173074 (targeting FGFR1) showed some promising activity, without evidence of cross-resistance with CDDP. Although these drugs were not further analyzed in combined treatments with CDDP, the obtained findings suggest that targeting protein kinases that influence DNA repair activities may indicate new promising therapeutic perspectives in OS, demanding for additional investigation. This perspective is particularly interesting because there are many protein kinase inhibitors in various stages of clinical development worldwide and the majority of them are used for cancer treatment (29).

Our results can also be the basis for further *in vitro* and *in vivo* studies aimed to improve the translation of these finding into the clinic. Development of 3D *in vitro* models may provide additional insights about the efficacy of these drugs against a tumor mass. Assessment of the efficacy of these agents in patient derived xenograft (PDX) models may further support their clinical use. All these activities are presently planned and will focus on the drugs screened and highlighted by this study.

CONCLUSIONS

In high-grade OS, when patients fail to respond to first-line treatment and relapse, therapeutic options and drugs effective for rescue chemotherapy protocols are scarce, also because resistance mechanisms developed against first-line chemotherapeutic drugs can also be responsible for reduced responsiveness to the agents used in the subsequent regimens.

Inhibition of DNA repair can be considered as a promising treatment strategy to enhance the efficacy of currently available DNA damaging drugs.

There are several genes and proteins involved in modulating the cellular response to DNA damage, each one may serve as target to enhance the efficacy of conventional therapeutic modalities. The current efforts in the development and deployment of several classes of DNA repair targeting compounds justify the hope to achieve new tailored treatment approaches through the use of these inhibitor drugs, which may ultimately drive toward innovative regimens aimed to improve patient outcomes.

The evidence emerged within this study about the possibility of successfully combining CDDP with drugs targeting DNA repair factors or protein kinases involved in these processes may indeed indicate new therapeutic options for specific OS patient cohorts, who have reduced cure probabilities.

DATA AVAILABILITY STATEMENT

Datasets generated and analyzed in this study will be made available by the authors to qualified researchers, upon justified request.

AUTHOR CONTRIBUTIONS

ET, MF, CH, and MS designed the study. MS coordinated the study and drafted the manuscript, which was revised by all authors, who approved it for publication. ET, MF, AF-R, MP, and FM carried out the cell culture-based experiments and the *in vitro* drug testing. ET, MF, SV, and AF-R performed the gene silencing screening. ET and MF carried out the DNA damage evaluations. ET, MF, CH, and SL took part in the molecular biology and western blot studies. ET, MF, CH, MP, and MS performed the statistical analyses. All authors have made a substantial, direct, and intellectual contribution to the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00331/full#supplementary-material

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SYSTEMATIC REVIEW
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Phase-II Trials of Pazopanib in Metastatic Neuroendocrine Neoplasia (mNEN): A Systematic Review and Meta-Analysis

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Background: Several phase-II trials have been designed to evaluate tyrosine kinase inhibitors (TKIs), in particular, pazopanib in neuroendocrine neoplasia (NEN), but its efficacy has not yet been demonstrated in a randomised-controlled Phase III trial. A systematic review of the published clinical trials of metastatic NEN patients could reduce the possible bias of single phase II studies. The present systematic review focuses on the efficacy and safety of pazopanib in patients with metastatic and locally advanced NEN.

Methods: A systematic search in the major databases Medline/PubMed, Cochrane and Embase and in supplementary material from important international Meetings was performed to identify publications on pazopanib for the treatment of neuroendocrine neoplasia. English language was defined as a restriction. Four authors of the present review independently performed the study selection, assessed the risk of bias and extracted study data. Four published clinical trials and 2 abstracts were identified. One trial was excluded because the topic was Von-Hippel Landau disease and one abstract was eliminated because of the lack of information on meeting proceedings.

Results: In all of the trials pazopanib was orally administered at a dose of 800 mg daily continuously with a 28-day cycle. The intention-to-treat population for efficacy was composed of 230 patients with a median age of 62 years. The partial response rate was 10.7% (95% confidence interval 2.6–20.5). The rate for stable disease was 79.6% (range: 61.7–92.1%) with a disease control rate (DCR) of 90.3%. Progressive disease was reported in 9.7% (range 5.2–17.6) of patients. No complete responses were observed. Median progression-free survival was 11.6 months (95% CI: 9.2–13.9). Overall survival from all the trials was 24.6 (95% CI: 18.7–40.8) months. Severe adverse events (grade III–IV) included hypertension 31%, 16% increase in AST/ALT, diarrhoea 10% and fatigue 10%.

Conclusions: Pazopanib monotherapy achieved a DCR of 90.3% in patients with locally advanced and/or metastatic neuroendocrine neoplasia, with an overall response rate comparable to other TKIs and mTOR inhibitors and a safety profile similar to that of drugs of the same class.

Keywords: pazopanib, neuroendocrine neoplasia, neuroendocrine tumours, review, carcinoid

INTRODUCTION

Rationale

Lung and gastroenteropancreatic (GEP) neuroendocrine tumours (NETs) are a heterogeneous group of malignancies derived from neuroendocrine cell compartments in various organs (1). A significant increase in the incidence of NETs over time has been reported ranging from 2.5 to 5 cases per 100,000 in Caucasian population (2–5). In unresectable or metastatic NETs, systemic treatment options are limited but in recent years there has been a renewed interest in expanding the therapeutic armamentarium (6). In particular, whilst in GEP-NETs the activity and safety of several compounds has been explored, in lung NETs only few drugs have been tested and the choice of treatment is often based on GEP-NET studies (7, 8).

NETs have been identified as hypervascular tumours. Vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) are usually overexpressed and are associated with poor prognosis (9). However, a modest clinical activity with bevacizumab, a monoclonal antibody targeting VEGF, has been observed in advanced neuroendocrine tumours in phase II studies (10, 11). In a phase III trial, sunitinib showed a superior efficacy to placebo in terms of progression-free survival (PFS) (11.4 vs. 5.5 months) leading to FDA and EMA approval for use in patients with advanced pancreatic NETs (pNETs) (12).

Pazopanib is an oral multitargeted tyrosine kinase inhibitor acting through VEGFR types 1–3, fibroblast-derived growth factor receptors (FGFR 1, 3, and 4), platelet-derived growth factor receptors α and β, and stem-cell factor receptor (c-Kit) (13, 14). Studies *in vitro* have shown that pazopanib inhibits ligand-induced autophosphorylation of VEGFR-2 PDGF-induced phosphorylation of c-Kit and PDGFRβ and VEGF-induced proliferation (13). *In vivo* pazopanib is known to inhibit FGF- and VEGF-induced angiogenesis in mouse models and has shown antitumour activity in different human models of solid tumours (15).

In one phase I trial, a patient with unknown primary neuroendocrine tumour obtained a partial response (PR) from treatment with pazopanib (16). Nevertheless, there are limited and non-conclusive data on the efficacy of tyrosine kinase inhibitors (TKIs) in both pNETs and non-pNETs, especially in those originating from the colorectum and small intestine where the incidence of the disease is high (6, 17).

Objectives

The aim of this systematic review was to evaluate the published studies assessing the activity and safety of pazopanib in patients with metastatic NEN (mNEN).

Research Questions

- Activity of pazopanib in patients with mNEN
- Safety of pazopanib in patients with mNEN
- Role of pazopanib in the therapeutic scenario of mNEN.

METHODS

Study Design

We report the results of a phase II systematic review and meta-analysis on the activity and safety of pazopanib in patients with mNEN. This study was performed according to PRISMA guidelines (18, 19)(see **Supplementary Materials**). The quality of included studies was assessed using the Downs and Black checklist (D&B checklist), which is appropriate for both randomised and non-randomised clinical trials. This checklist consists of 27 items distributed between five subscales. The total maximum score is 32. A study scoring 16 or more is ranked as a high quality study (20).

Participants, Interventions, Comparator

We included all articles with prospective data on mNEN in adult patients treated with pazopanib. All of the studies included were in the English language.

Systematic Review Protocol

We developed a protocol that had pre-specified objectives, eligibility criteria, data of interest, search strategy, and analysis plan. The present systematic review was registered in the PROSPERO database.

Data Source Study Section and Data Extraction

A search of the major databases Medline/PubMed, Cochrane and Embase was performed to identify publications on pazopanib for the treatment of neuroendocrine neoplasia (21). Search terms used included "pazopanib" and/or "neuroendocrine." A supplementary search of congress abstracts published between 2014 and 2019 was also carried out for the annual meetings of the American Society of Clinical Oncology (ASCO), ASCO Gastrointestinal Symposium (ASCO-GI), and European Society for Medical Oncology (ESMO). A manual search of the references of retrieved articles for additional relevant publications was also performed. References from systematic reviews and meta-analyses were screened to ensure search sensitivity (**Figure 1**).

Two authors independently conducted a preliminary screening of reports by reading titles and abstracts. Duplicate publications were removed. All identified citations were reviewed and those considered unrelated were excluded. The full texts of potentially relevant articles were then downloaded for the

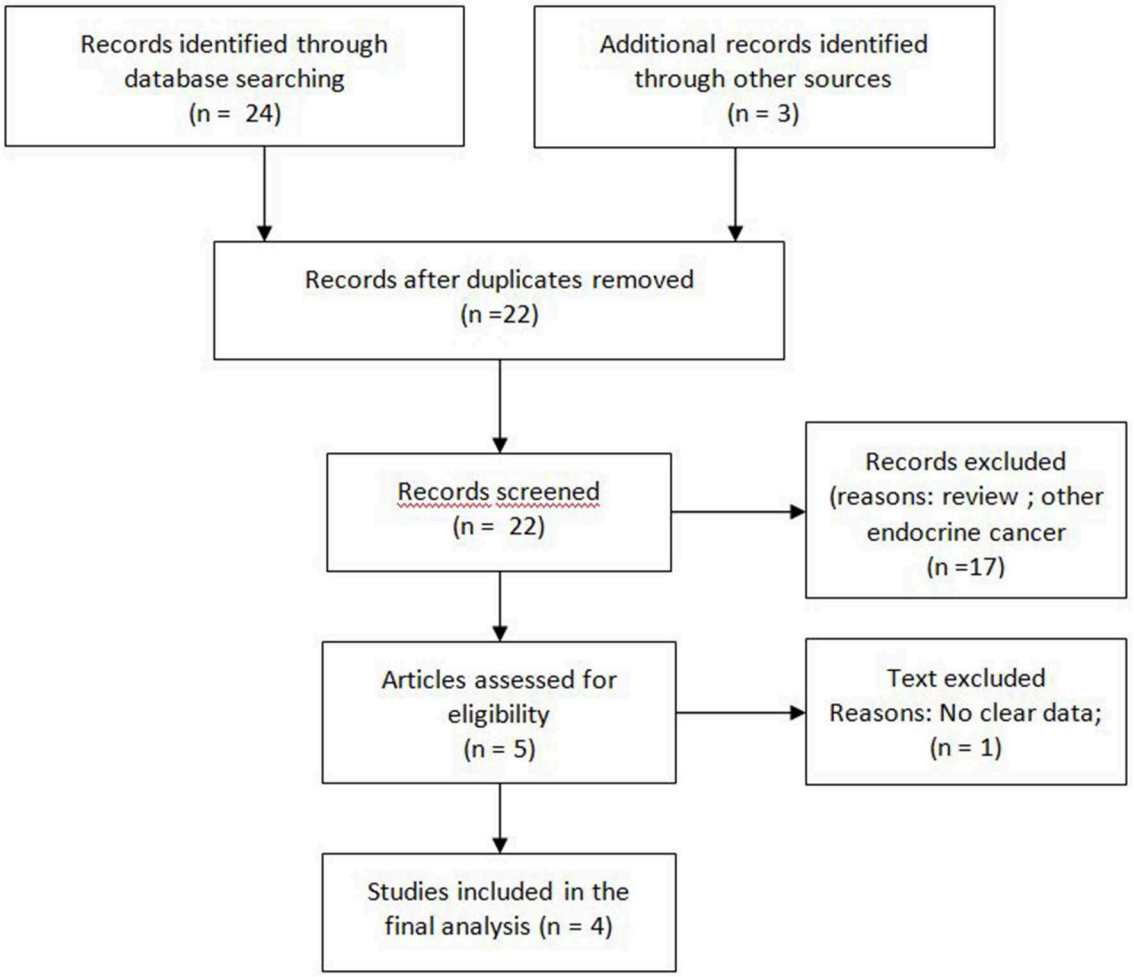

FIGURE 1 | Flow diagram of search methods.

second round of screening. When disagreement existed, two authors discussed with a third reviewer to reach a final decision. Data from included studies describing the population treated as well as treatment efficacy and toxicity parameters were extracted and pooled.

For each study, the following data were collected and tabularised for the analysis: year of publication, name of the first author, area of study; study design; baseline characteristics of patients included; intervention including regimens, dosages and cycles; outcomes including overall response rate (ORR), disease control rate (DCR), progression-free survival (PFS) and overall survival (OS); toxicities including those of a haematological and non-haematological nature.

Statistical Analysis

For survival primary endpoints, meta-analyses usually deal with hazard ratios which can only be obtained when the experimental treatment is compared to a control treatment. However, single-arm exploratory phase II studies aimed at estimating the survival curve are far from rare, especially in the area of rare tumours. In this scenario, the PFS and OS curves are usually summarised by medians and accompanied by their 95% confidence interval (95% CI), as is the case of the present review. Following the method used by McGrath et al., pooled estimates were obtained as the median of the study-specific PFS and OS medians (22), whereas the corresponding 95%CIs were obtained as the $1/2 \pm \min\left\{1/2,\ z_{0.975}/(2\sqrt{K})\right\}$ quantiles of the k observed study medians, with z_α the α quantile of the standard normal distribution.

Heterogeneity between the median PFS and OS of studies was evaluated using the I^2 index that quantifies values higher than 50%, indicating sizable heterogeneity. Furthermore, the Cochran Q-test was used to infer the null hypothesis between study homogeneity at a significance level $\alpha = 0.10$.

All of the statistical analyses were performed with the statistical language R version 3.6.1. The metamedian package was used to compute the pooled estimates and their 95% CIs, while the *ad hoc* code was used to compute the I^2 index and infer homogeneity via the Cochran Q-test.

RESULTS

Study Selection and Characteristics

The systematic search of the literature identified four studies meeting selection criteria (**Figure 1**): three peer-reviewed journal publications [(23–25) and one conference abstract/poster (24)]. Briefly, one randomised and three non-randomised prospective phase II studies included a total of 304 patients of whom 74 were treated with placebo. Three studies were multicentric and only one was monocentric (23). Two studies had an independent review (23, 26). All the studies were of high quality according to the D&B checklist. Patient number, tumour histology (grade and primitive site), Eastern Cooperative Oncology Group Performance Status (ECOG PS) and other characteristics of each study are shown in **Table 1**.

Summary of Findings

Population Characteristics

A total of 304 patients were included in the selected trials. Progressive disease during other previous treatment was found at

TABLE 1 | Principal characteristics of the Phase II studies.

References	Country	Phase	Type of publication	Randomised vs. placebo	No. of patients	Grade	Primitive site	ECOG PS	Biomarker evaluation	Setting
Ahn et al. (23)	Asia	II	Full Text	No	37	G1 G2 G3	Pancreas GI Lung Unknown Other	0–1	No	Metastatic only
Phan et al. (24)	US	II	Full Text	No	52	G1 G2	GI Pancreas	0–1	No	Metastatic/locally advanced
Grande et al. (25)	Europe	II	Full Text	No	44	G1 G2	Pancreas GI Lung Unknown Other	0–1	Yes	Metastatic/locally advanced
Bergsland et al. (26)	US	II	Abstract	Yes	171	G1 G2 UK	Pancreas GI Lung Other	0–1	Yes	Metastatic/locally advanced

ECOG PS, Eastern Cooperative Oncology Group Performance Status; GI, gastrointestinal.

TABLE 2 | Sample sizes and median PFS and OS in months along with their 95% confidence intervals (CIs).

Study	Stratification	Sample size	Median PFS (95% CI)	Median OS (95% CI)
Ahn et al. (23)	–	37	9.1 (4.9, 13.3)	–
Phan et al. (24)	Pancreatic NETs	32	14.4 (5.9, 22.9)	25.0 (15.5, 34.4)
	Carcinoid tumours	20	12.2 (5.3, 19.0)	18.5 (15.0, 22.0)
Grande et al. (25)	–	44	9.5 (4.8, 17.1)	24.1 (20.0, 28.3)
Bergsland et al. (26)	–	97	11.6 (NA, NA)	41.3 (NA, NA)

PFS, progression-free serviva; OS, overall survival; NA, not applicable.

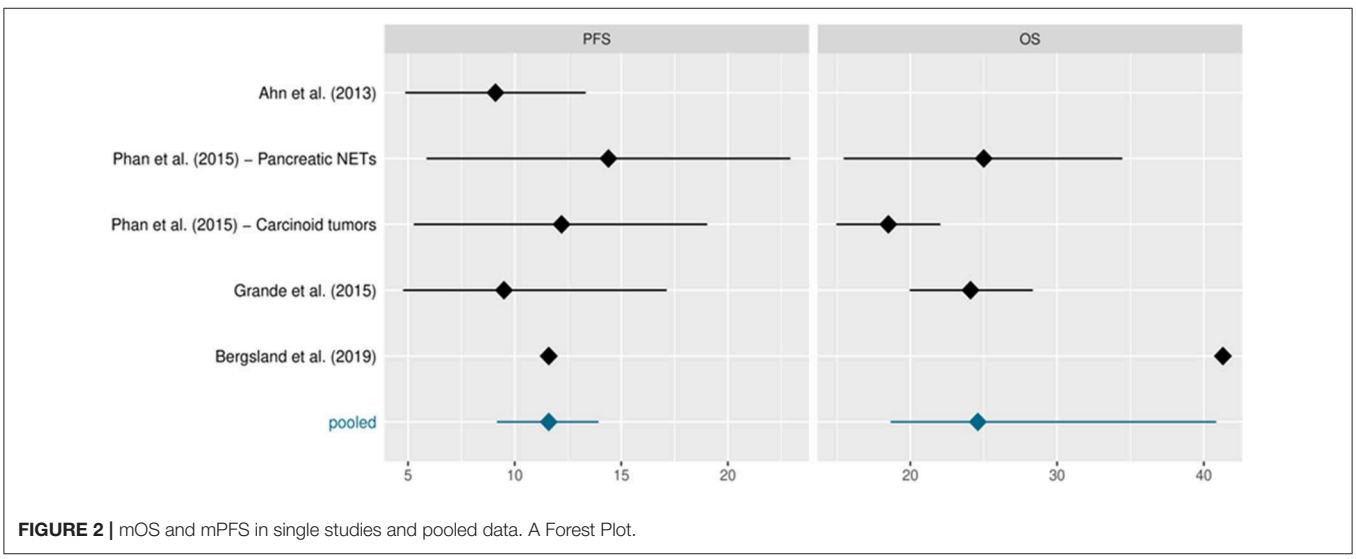

FIGURE 2 | mOS and mPFS in single studies and pooled data. A Forest Plot.

the time of enrolment in 283 (93.1%) patients. Previous therapies included somatostatin analogues (SSA) in 177 (58.2%) patients, other TKIs in 16 (5.2%), everolimus in 25 (8.2%), both TKI and everolimus in 8 (2.6%), chemotherapy in 56 (18.4%), hepatic locoregional treatment in 38 (12.5%) and other non-specified treatments in 19 (6.2%). One hundred fourteen (37.5%) patients had tumours of gastrointestinal (GI) origin, while the remaining (190, 62.5%) had NEN of lung, pancreatic and unknown origin. The majority of patients (76.3%) had grade 1 or 2 NEN and 15 (5%) had grade 3 NEN. Tumour grade was unknown in 58 (18.7%) patients. Seventy patients had a functioning tumour (23%). SSAs were administered together with pazopanib in 230 (75.6%) patients.

Clinical Outcomes

The intention-to-treat population treated with pazopanib comprised 230 patients, excluding 74 patients in the Bergsland study who were treated with placebo. **Table 2** shows the study sample sizes or those of the various study arms when reported in the protocol. Median PFS and OS, reported in months, are also included along with their 95%CIs, whenever available. The data derive from single-arm phase II studies, with the exception of Bergsland et al.'s study (26) which was a phase II randomised controlled trial (for the purposes of this review we only considered the experimental pazopanib arm). Phan et al. (24) reported distinct median PFS and OS for patients with pNETs and carcinoid tumours, respectively. Ahn et al. (23) did not evaluate OS and therefore the pooled median was based on the remaining values. Bergsland et al. (26) did not report 95%CIs for PFS or OS. A response to pazopanib was reported in 186 patients. The studies registered stable disease (SD) in 148 (79.5%; range: 95% CI 61.7–92.1%) patients, partial response (PR) in 20 (10.7%; 95% CI, range 2.6–20.5%) and progressive disease (PD) in 18 (9.7%; 95% CI range: 5.8%−17.6%). No complete responses were observed. The DCR was 90.3%. Median PFS and OS from all trials was 11.6 (95% CI: 9.2, 13.9) and 24.6 (95% CI: 18.7, 40.8) months, respectively (**Figure 2** and **Table 2**).

Side-Effects

Safety outcomes are presented in **Table 3**. The rate of G1-G4 toxicities experienced was 70%. The most frequent adverse events were fatigue (65%), hypertension (50%), neutropoenia (26.5%), mucositis (16%), H&F syndrome (15.6%), thrombocytopoenia (15.2%), anaemia (9.1%) and proteinuria (4.7%). The rate of grade (G)3-4 toxicity was 45.2%. The most frequent G3-G4 adverse event was hypertension (15.6%).

Risk of Bias

The studies included in this systematic review were phase II studies. The fact that we included the survival estimates of the pazopanib arm in Bergsland et al.'s study (26) eliminates the potential drawbacks of considering trials with different designs. Similarly, the study by Phan et al. (24) reported distinct median PFS and OS for both pNET and carcinoid tumour arms. We considered these values in the meta-analysis because they came from different studies. The relative similarity between median

TABLE 3 | List of side-effects grouped by grade.

	Grade 1-2 no. (%)	Grade 3-4 no. (%)	All grade no. (%)
Haematological side-effects			
Anaemia	16 (80.0)	4 (20.0)	20 (100)
Neutropoenia	40 (87.0)	6 (13.0)	46 (100)
Thrombocytopoenia	34 (97.1)	1 (2.9)	35 (100)
Non-haematological side-effects			
Abdominal pain	45 (91.8)	4 (8.2)	49 (100)
Alkaline phosphatase	9 (90.0)	1 (10.0)	10 (100)
Alopoecia	7 (100)	0	7 (100)
Anorexia	34 (94.4)	2 (5.6)	36 (100)
AST/ALT increase	124 (83.8)	24 (16.2)	148 (100)
Asthenia	30 (81.1)	7 (18.9)	37 (100)
Blood bilirubin increase	29 (93.5)	2 (6.5)	31 (100)
Confusion	0	1 (100)	1 (100)
Constipation	8 (100)	0	8 (100)
Dehydration	0	1 (100)	1 (100)
Diarrhoea	114 (89.8)	13 (10.2)	127 (100)
Dizziness	7 (100)	0	7 (100)
Oedema	9 (100)	0	9 (100)
Erythema	5 (100)	0	5 (100)
Fatigue	103 (89.6)	12 (10.4)	115 (100)
Fever	7 (100)	0	7 (100)
H&F syndrome	34 (94.4)	2 (5.6)	36 (100)
Hair depigmentation	11 (100)	0	11 (100)
Headache	17 (100)	0	17 (100)
Hepatotoxicity	8 (53.3)	7 (46.7)	15 (100)
High GGT	5 (100)	0	5 (100)
High LDH	7 (100)	0	7 (100)
Hyperglycaemia	15 (83.3)	3 (16.7)	18 (100)
Hypertension	79 (68.7)	36 (31.3)	115 (100)
Hypertriglyceridaemia	0	1 (100)	1 (100)
Hypocalcaemia	7 (100)	0	7 (100)
Hypokalaemia	1 (50)	1 (50)	2 (100)
Hypomagnesaemia	9 (100)	0	9 (100)
Hypophosphataemia	5 (100)	0	5 (100)
Hyporexia	7 (87.5)	1 (12.5)	8 (100)
INR increase	7 (100)	0	7 (100)
Insomnia	5 (100)	0	5 (100)
Mucositis	37 (100)	0	37 (100)
Nausea	123 (96.1)	5 (3.9)	128 (100)
Pain	19 (90.5)	2 (9.5)	21 (100)
Pancreatitis	0	1	1 (100)
Proteinuria	7 (63.6)	4 (36.4)	11 (100)
Pruritus	4 (100)	0	0 (100)
Rash	18 (94.7)	1 (5.3)	19 (100)
Skin and subcutaneous tissue disorders	6 (100)	0	6 (100)
Skin hypopigmentation	7 (100)	0	7 (100)
Drowsiness	6 (100)	0	6 (100)
Thromboembolic events	0	1 (100)	1 (100)
Urinary tract infection	0	1 (100)	1 (100)
Vomiting	41 (9.1)	4 (8.9)	45 (100)

survival estimates, especially for PFS, partially safeguarded against extreme results.

DISCUSSION

Summary of Main Findings

Phase II trials provide a valuable insight into diseases, treatment efficacy and safety, especially in settings where is it difficult to carry out large randomised phase III clinical studies i.e., in the area of rare tumours. In a phase II setting, surrogate endpoints are usually taken into consideration as an early sign of drug activity and can facilitate the decision-making about whether to proceed with phase III testing. Sunitinib is still the only approved TKI for the treatment of advanced pNETs, showing a clear impact in terms of PFS and ORR. However, despite an initial benefit, sunitinib inevitably loses its effectiveness because of the activation of downstream pathways that induce resistance, leading to increased invasiveness and metastasis (27, 28). Peptide radionuclide receptor therapy (PRRT), chemotherapy and everolimus are other therapeutic options, but patients progressing on these treatments are left with few, if any, alternatives (29).

To the best of our knowledge, the present systematic review is the first to assess phase II literature on the effectiveness of pazopanib in NEN. Pazopanib achieved a DCR of 91.3% and a median PFS and OS of 11.6 and 24.6 months, respectively, superior to results of other targeted therapies in the same setting (DCR ranging from 72 to 84% and median PFS of 11–12.6 months) (12, 30–32). Of note, although half of the patients were pretreated, the pazopanib activity was maintained. Furthermore, the addition of SSAs would appear to promote a synergistic effect, increasing the DCR in this patient subgroup. A recently published network meta-analysis supports this hypothesis of the additional effect of the SSA combination with other therapies (33).

Recently, some phase II trials have been carried out to obtain a breakthrough therapy designation from the regulatory authorities for tumours whose therapeutic armamentarium is limited (34, 35). However, the interpretation of data from phase II trials has faced difficulties because of the lack of a control group, hampering direct and scientifically robust comparisons, and small patient samples. The added value of a phase II systematic review and meta-analysis could help to overcome the problem of sample size for patients treated in single trials and amplify the efficacy data of a drug evaluated prospectively in small studies.

Safety profile is also crucial factor. The results of the present review indicate that pazopanib carries a substantial risk of adverse events that can affect patient quality of life. However, the incidence of G3-G4 toxicities reported in the largest and most recent trial was 15% lower than that of previous studies. These data suggest an increasing familiarity with pazopanib over time due to its ł widespread use, and a better management of it side-effects. Overall, given that pazopanib seems to have a disease control rather than curative effect in NENs, quality of life should be take in consideration in future prospective studies.

Limitations

This study has some limitations. We conducted a comprehensive literature search with a sensitive search algorithm and an extensive manual search of reference lists and conference proceedings. However, we were unable to obtain additional unpublished data and are aware that a substantial amount of information is not available to the public. Another limitation is the low number of phase II clinical trials with different types of study design and populations included. Despite this, we believe that our results could provide important indications for the design of future dedicated clinical trials on NETs to underline the importance of head-to-head comparisons and the correct patient setting. Furthermore, the addition of SSAs to experimental drugs could be taken into consideration when designing dedicated trials on NETs.

CONCLUSIONS

Overall, our current pooled analyses of data on pazopanib in phase II studies are essentially consistent with the data available for other approved drugs. Surprisingly, although pazopanib was one of the first and most widely studied TKIs in neuroendocrine tumours, it has not moved to phase III. For this reason and because of the rarity of the disease, we decided to further investigate pazopanib activity in terms of DCR and mPFS. The clinical information available supports the use of pazopanib for the treatment of metastatic neuroendocrine tumours of different origin, especially those of the gastrointestinal tract.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AB, CL, and TI conceived the idea for the study and drafted the article. GD, VF, CC, and FR were responsible for data acquisition. SN, SS, AV, and AD performed the meta-analysis and co-drafted the manuscript. CS, GM, and LM assessed the quality of the manuscript independently through the Downs and Black checklist. All authors read and approved the present version of the paper for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00414/full#supplementary-material

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MINI REVIEW
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Pharmacogenomics Biomarkers of Soft Tissue Sarcoma Therapies

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Soft tissue sarcomas (STS) are heterogeneous rare malignancies comprising ∼1% of all solid cancers in adults and including more than 70 histological and molecular subtypes with different pathological and clinical development characteristics. Over the last two decades, the increased knowledge of the new molecular and genomic mechanisms of different STS histotypes allowed for a reclassification of these tumors and consequently to the development of novel chemotherapeutic agents. Generally, surgery, in combination with radiotherapy only in selected cases of localized disease, represents the most common treatment of primary STS, whereas the principal treatment modality for locally advanced or metastatic disease is first-line chemotherapy. The principal treatment for the preponderance of STS patients is usually an anthracycline (epirubicin and doxorubicin) in monotherapy or in combination with other drug novel chemotherapeutic agents. However, survival for treated patients with metastatic disease is poor, and a 2-years survival rate is about 30%. In this scenario, Pharmacogenomics (PGx) biomarkers that can predict drug response play an important role in the improvement of molecular diagnostics in clinical routines and contribute to elucidating the genetic basis for the differences in treatment efficacy and toxicity among STS patients. This review focuses on recent insight in the PGx biomarkers that have been described to modulate responsiveness and toxicity parameters of conventional and new chemotherapeutics drugs in several STS histotypes.

Keywords: soft tissue sarcoma (STS), pharmacogenomics (PGx), resistance and mutation, genetic variation, somatic mutation, toxicity

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INTRODUCTION

Soft tissue sarcomas (STS) are heterogeneous rare malignancies representing of about 1% of all solid tumors in adult and including more than 70 histological and molecular subtypes with a multiplicity of pathogenic and clinical development features (1–4). STSs origin from mesenchymal cells of a variety of tissue lineage, including adipose, muscle, fibrous cartilage, and vasculature. Among this heterogeneousness, the most common STS is represented by liposarcoma (LPS), accounting for one-fourth of all extremity STS and half of retroperitoneal STS (5). Different histotypes of high-grade STS frequently diagnosed include leiomyosarcoma, synovial sarcoma, undifferentiated pleomorphic sarcoma, and malignant peripheral nerve sheath tumors.

Over the last two decades, the increased knowledge of the new primary molecular and genomic mechanisms of different STS histotypes allowed to a reclassification of these tumors and consequently to the discovery of innovative chemotherapeutic agents (6). Overall, sarcomas can be classified in two comprehensive genetic groups depending on the chromosomal aberration occurring in the genome: those harboring specific genetic alterations like activating mutations

and/or translocations showing simple karyotypes (which represent almost 30% of all sarcomas) and those with more complexity on karyotypes (7). The gene fusions resulting from specific translocations encoding chimeric transcription factors affecting transcriptional regulation of target genes are frequently detected in sarcomas, while others encode chimeric growth factors or protein tyrosine kinases (8).

Despite the prominent progress in discovering genetic aberrations and their functions in STS, the major therapeutic modality for most local recurrence and metastatic sarcomas remains cytotoxic chemotherapy. Generally, surgery, in combination with radiotherapy only in selected cases of localized disease, represents the most common treatment of primary STS, whereas the principal treatment modality for locally advanced, or metastatic disease is chemotherapy. First-line drug protocol for the preponderance of STS patients is usually an anthracycline (epirubicin and doxorubicin) alone or in combination with another drug (9, 10). However, survival for treated patients with metastatic disease is only 14-17 months, and 2-years survival rate is about 30%.

Therefore, there is an urgent need to develop novel treatments and find biomarkers that can help physicians to identify patients who are possible good responders or resistant to specific therapies and predict individual predisposition to toxicity reactions associated with therapies.

Differences in pharmacological response to drugs represent the most common cause of patient morbidity and mortality. From this specific point of view, pharmacogenomics (PGx) biomarkers that can predict drug response play an important role in the improvement of molecular diagnostics in clinical routines and contribute to elucidating the genetic basis for the differences in treatment efficacy and toxicity among patients. Moreover, PGx markers predicting efficacy or risk to develop adverse drug reactions (ADRs) are commonly positioned in transporters, drug-metabolizing enzyme genes, drug targets, or HLA alleles.

Genetic variability harboring in the germline genome of the patient can influence systemic pharmacokinetics and pharmacodynamics of the treatments, acting as prognostic biomarkers for drug-induced toxicity and treatment efficacy. Instead, the aberrations in cancer somatic genome mostly function as drug targets and they can be used to select treatment or to be predictive of response to treatment (11).

Very penetrant predisposed mutations and frequent genetic variants particularly single-nucleotide polymorphisms (SNPs) which heredity pass between the generations represent the mostly germline variations that are considered as useful biomarkers for ADR and drug response. Contrary, due to exposure to chemotherapeutics that likely act through damage to DNA, cells could acquire randomly somatic mutations that are potentially used as drug targets (12, 13).

Thanks to significant improvements in biotechnology and bioinformatics knowledge, genomic research quickly advanced from investigations based on modifications at the single gene level to studies on the whole-genome scale using extensive genotyping, and Next Generation Sequencing (NGS) methods. These new methodologies considerably decrease sequencing times and costs and allow early detection of disorders and identification of pharmacogenomics markers to customize treatments (12, 14).

Candidate gene methods are performed to recognize most of the germline variations while genome-wide association (GWAS) approach is archived sequencing up to a large number of SNPs. On the other hand, somatic mutations from cancer genomes are analyzed through NGS technique that uses the concurrent sequencing of a huge number of DNA parts to create an enormous pool of genomic arrangement information. This procedure allowed genotyping a selected number of the gene of interest (gene panel), the complete exome or the whole genome.

In this review, we outline recent studies on PGx biomarkers that have been described to modulate responsiveness and toxicity parameters of conventional and new option chemotherapeutics drugs in several STS histotypes (**Tables 1**, **2**).

GERMLINE VARIANTS AS POTENTIAL BIOMARKERS FOR DRUG RESPONSE

Several germline biomarkers could impact on effectiveness of therapies and survival in STS patients and may be useful to stratify patients liable to develop treatment-associated toxicities.

One of the new therapeutic alternatives among the few options of STS treatments is trabectedin (Yondelis) a marine-derived compound extracted from the Caribbean Sea squirt *Ecteinascidia turbinate*.

In phase III clinical trial in advanced leiomyosarcoma and liposarcoma patients showing progression disease after anthracycline-based chemotherapy, trabectedin significantly increases disease control respect to conventional dacarbazine treatment (15).

Several studies confirmed that the cytotoxic activity of trabectedin toward cells has been associated with the peculiar capacity to modify positively the tumor microenvironment and exert strong immunomodulatory effects (5, 16). The main antiproliferative mechanism consists of transcription regulation and DNA repair systems, including transcription-coupled nucleotide excision repair (TC-NER), homologous recombination repair (HRR) and, DNA repair genes such BRCA1 (BReast-CAncer susceptibility gene 1) and BRCA2. Additionally, the association of BRCA mutational status with improved clinical response to trabectedin explains the specific sensitivity of STS patients to this drug. Several clinical studies confirmed an improved prognosis and overall survival in patients carrying germline mutation or absence of BRCA compared to non-carriers (17). *Italiano et colleagues* have pointed out the relationship of precise haplotypes associated with trabectedin sensitivity to specific SNPs within the BRCA1 gene (18). In this study, advanced STS harboring at least one AAAG allele on BRCA1's haplotype displayed a statistically significantly longer progression-free survival (PFS) and overall survival, compared with STS without AAAG allele. Moreover, in 29% of human uterine leiomyosarcoma one of the histotypes more responsive to trabectedin, BRCA1 protein was not express (19).

TABLE 1 | Germline variants biomarkers in STS therapies.

Drug	Gene	Germline biomarker	STS histotype	References
Trabectedin	BRCA1	AAAG rs16941	Advanced STS	(18)
	BRCA2	(LOH) rs80359030	Uterine Stromal Sarcoma	(20)
Imatinib	VEGFR2	AA rs1870377	GIST	(27)
	VEGFA	AA rs1570360		
	SLCO1B3	T rs4149117		
Sunitinib	POR	TT rs1056878	GIST	(28)
	SLCO1B3	T rs4149117		
	SLC22A5	C rs2631367		

TABLE 2 | Pharmacogenomics (PGx) somatic biomarkers in STS therapies.

Drug	Gene	Somatic variation	STS histotype	References
Pazopanib	TP53	mutation	Advanced STS	(33)
Gemcitabine	hENT1	high expression	Leiomyosarcoma; Angiosarcoma	(38)
Trabectedin	BRCA1	low expression	Leiomyosarcoma; Myxoid Liposarcoma; Liposarcoma; Osteosarcoma; Synovial Sarcoma; Uterine Leiomyosarcoma; Ewing Sarcoma	(39)
	ERCC5/XPG	high expression	Leiomyosarcoma; Myxoid Liposarcoma; Liposarcoma; Osteosarcoma; Synovial Sarcoma; Uterine Leiomyosarcoma; Ewing Sarcoma	
Conventional chemotherapies	CD109 (TGF-β)	high expression	Myxofibrosarcoma	(44)
Conventional chemotherapies	RB1; CDKN2A; CDKN2B; CCND1; CDK6; TP53	mutation	Myxofibrosarcoma	(45)
	KRAS	amplification		

A remarkable clinical case study describes a patient with advanced uterine stromal sarcoma with bone and hepatic metastases carrying a specific BRCA2 germline variant. The authors revealed a complete rapid response following trabectedin treatment linking this positive effect to the loss of heterozygosity (LOH) of the mutated BRCA2 gene. These analyses corroborate the assumption that different DNA repair defects existing in tumors positively conditioned the response to trabectedin and that BRCAness malignant genotype is significant in influencing the effectiveness of treatment including trabectedin (20).

Gastrointestinal stromal tumors (GIST) are the most prevalent tumors of the gastrointestinal tract origin from mesenchymal lineage (21). Mutation in tyrosine protein kinase KIT and platelet-derived growth factor receptors (PDGFRA) genes are present in 75–80% and 5–10% of GISTs, respectively with their consequent constitutive activation. Imatinib, sunitinib and regorafenib, TKIs that inhibit KIT/PDGFRA tyrosine kinase, demonstrated efficacy in unresectable and/or metastatic GIST (22). In almost 80% of patients with advanced or metastatic GIST treated with imatinib (400 mg per day), quick partial response or stable disease was observed for ∼18–36 months, with some patients in therapy for 10 years. Despite the greater clinical advantage of these drugs, PFS is variable due to a frequent resistance mechanism depending on mutational board of KIT/PDGFRA genes. Commonly, GISTs harbor KIT mutation in exon 11 and less frequently in exon 13 in imatinib-naïve patients, while exon 9 mutation reduces sensitivity and the rare KIT exon 17 mutations (e.g., D816V) exert resistance to imatinib. Moreover, the common D842V mutation in PDGRFA gene is correlated to imatinib, sunitinib, and regorafenib resistance, whereas wild-type GISTs negative for KIT/PDGRFA mutations are insensitive to imatinib (23–25). Thus, it is crucial to find novel prognostic biomarkers to stratify patients with improved risk for disease progression during imatinib therapy. Analysis of SNPs variant in *VEGFRA2*, *VEGFA,* and Solute Carrier Organic Anion Transporter Family Member 1B3 (*SLCO1B3)* display a correlation of these SNPs with PFS in patients with advanced GIST receiving imatinib (26). Genetic variant analysis of 36 SNPs in 18 genes performed in patients with advanced GIST treated with imatinib demonstrated a correlation between worse PFS and VEGFR2, VEGFA, and *SLCO1B3* carrying specific genotype listed in **Table 1** (27).

Association of SNP and outcome of GIST patients cured with sunitinib was also highlighted by *Kloth and colleagues*. In

this study, PFS and OS in 127 patients with advanced GIST treated with sunitinib were associated with 49 SNPs involved in the pharmacokinetic and pharmacodynamic pathway of this TKI. More specifically, PFS was significantly extended in carriers rs1056878 (TT genotype) in *Cytochrome p450 oxidoreductase* (*POR*). Otherwise, the presence in patients carrying the T-allele in SLCO1B3 rs4149117, the CCC-CCC alleles in SLC22A5 haplotype, and the GC-GC alleles in the IL4 R haplotype were predictive for OS (28).

Pazopanib, currently approved for the treatment of different STS, is multitarget TKI exerting its clinical antitumor effects through inhibiting vascular endothelial growth factor receptor (VEGFR)-mediated angiogenesis and by directly blocking PDGFRs, fibroblast growth factor receptors (FGFRs), and KIT (29, 30). The results of the PALETTE study designed to compare the efficacy and safety of pazopanib with placebo in advanced pretreated STS, led to Pazopanib, approval as single-agent in patients with metastatic STS from non-adipocytic origin (31). One of the potentially serious consequences of TKI therapy usually described in patients following pazopanib therapy is hepatotoxicity. Recent data provide innovative understanding connecting the pazopanib-associated hepatotoxicity to an immune-mediated mechanism in some patients, demonstrating that HLA-B*57:01 allele carriage is correlated with elevated ALT values in these patients and identifying genetic PGx predicting liver damage (32).

SOMATIC MUTATION BIOMARKERS

Genetic analysis of STS shows low mutational load including predominantly by copy number changes (6). Whole-exome sequencing (WES) data analysis of 206 sarcomas of different histotypes identifies TP53, ATRX, and RB1 significantly mutated genes across sarcoma histotypes where TP53 mutations were most prevalent in leiomyosarcoma and RB1 mutations were seen in leiomyosarcoma, undifferentiated pleomorphic sarcoma, and myxofibrosarcoma (6).

A recent retrospective study reported the new early PGx markers related to response and toxicity of pazopanib therapy in advanced STS. In this study, application of NGS analysis performed to sequence several genes related to cancers in pretreatment tumor specimens from patients with advanced STS treated with antiangiogenic agents (pazopanib and sunitinib) (33), reveals the importance of TP53 and RB1 genes in modulating the outcome of TKI treatments. Although all loss-of-function mutational status of TP53 detected (missense mutation of DNA binding and/or tetramerization domain, or homozygous deletion) was not correlated to outcome of patients treated with pazopanib, *TP53* mutations were shown to have significant association with a longer PFS respect to *TP53* wild-type. Predictors factors of pazopanib effectiveness and toxicity in STS patients are associated also with modulation of cytokines and circulating angiogenic factors in serum (34). Indeed, PFS observed after 12 weeks of treatment was positively correlated to high levels of interleukin (IL)-12 and mitochondrial pyruvate carrier 3 (MPC3) levels at baseline, and negatively associate with low soluble VEGFR2 and high placental growth factor (PGF) levels.

Gemcitabine, in monotherapy or combined with docetaxel, has been usually approved in leiomyosarcoma (35) and angiosarcoma (36) treatments.

Intracellular uptake of prodrug gemcitabine into tumoral cells takes place through a transmembrane protein human equilibrative nucleoside transporter 1 (hENT1) (37). A recent retrospective analysis demonstrated that positive clinical outcome of leiomyosarcoma (PFS: 6.8 vs. 3.2 months; OS: 14.9 vs. 8.5 months) and angiosarcoma (PFS was 9.3 vs. 4.5 months; OS 20.6 vs. 10.8 months) patients treated with gemcitabine was linked to high hENT1 tumor expression levels (38). Thus, since the identification of molecular markers like hENT1 could predict gemcitabine efficacy in leiomyosarcoma and angiosarcoma patients, evaluation of hENT1 expression level would allow a better patient selection with a high possibility to benefit from this chemotherapy regimen.

Not only germline variants as discussed before but also somatic alterations in the homologous repair system are reported to be responsible for a deeper and longer activity of trabectedin in STS patients where drug response is inversely correlated with the BRCA1 mRNA levels (39). In this clinical report, the investigators established that low levels of mRNA BRCA1 expression statistically significant associate with an improved outcome of patients in terms of disease control rate (48 vs. 26%, $p < 0.01$) and longer median survival (15.4 vs. 7.1 months, $p < 0.002$). Interestingly, patients with decreased level of mRNA BRCA1 showed a better median PFS (4.7 vs. 2.0 months, $p = 0.002$) and a progression-free at 6-months (PFS-6) after treatment (43 vs. 23%, $p < 0.012$). Moreover, a significant correlation between increased responses to trabectedin treatment with high expression level of ERCC5/XPG complex was also observed in patients showing an improvement in term of disease control rate (56 vs. 36%, $p = 0.04$), median PFS (7.1 vs. 2.5 months, $p = 0.002$), and PFS after 6 months after trabectedin therapy (52 vs. 30%, $p = 0.01$). These data support the hypothesis of a direct association between DNA damage repair system functionality and responsiveness to trabectedin, differently from other DNA interacting agents.

In myxofibrosarcoma, a common adult STS characterized by a high local recurrence rate and infiltrative growth pattern surgery combined with neoadjuvant or adjuvant radiotherapy represent the standard care in localized disease (40–42). However, chemotherapy treatment is considered for metastatic myxofibrosarcoma despite the outcome remains very poor and identification of PGx markers is still limited (43).

Genotyping analysis in patient-derived MFS primary cultures demonstrated the promising role of surface glycoprotein CD109, a negative regulator of transforming growth factor-beta (TGF-β) pathway in the differential diagnosis of more aggressive high-grade myxofibrosarcoma identifying this marker as a possible therapeutic target (44). Moreover, in this study, the authors highlighted the value of TGF-β expression as an advantageous marker for chemotherapy efficacy and resistance. Indeed, in patient-derived colture cells of myxofibrosarcoma, the expression of TGF-β was negatively correlated to sensitivity to treatments.

In an extensive integrated genetic and epigenetic study of 99 myxofibrosarcoma performed by WES, RNA sequencing, and methylation analysis, a large number of driver genes were identified as potential drug targets and molecular prognostic factors in this STS histotype (45). This study demonstrated the association of the mutational board of cell cycle regulators (*RB1*, *CDKN2A*, *CDKN2B*, *CCND1*, and *CDK6*) with a worse overall survival as well as *TP53* alteration and *KRAS* amplification. Thus, considering as PGx markers in a specific subset of these tumors genetic alterations in the Rb pathway, comprising *CCND1* or *CDK6* amplification, these data will contribute to knowledge for the use of novel therapeutic approaches such as CDK4/6 inhibitors.

Besides genetic factors, epigenetic modifications of DNA together with miRNA regulation of gene expression have been linked to differences in drug response, through regulation key drug-metabolizing genes or increasing expression of drug efflux transporters (46–49).

The role of these biomarkers in mediating chemotherapy efficacy was underlined in eribulin-based therapies in STS patients. Eribulin mesylate is a microtubule inhibitor equivalent to halichondrin B derivate from the marine sponge *Halichondria okadai*. The inhibition of tubulin by eribulin induces G2/M cell-cycle arrest, disruption of mitotic spindles, and, finally, apoptosis. Patient-derived primary coltures of adipocytic and undifferentiated pleomorphic sarcoma demonstrated high sensitivity to eribulin (50, 51). Moreover, the antitumor activity of eribulin in metastatic STS patients was confirmed in recent EORTC 62052 phase II and III clinical trials (52–54). miRNA expression signature in 65 tumor samples from patients included in the EORTC phase II clinical trials indicated miR 106a, miR-17, and miR-34a as markers modulated in eribulin responders respect to non-responders STS patients, pointing out the role of these miRNA as useful tools for clinical practice to stratify patients that can really benefit from the eribulin treatment (55).

DISCUSSION

Pharmacogenomics studies of anti-cancer drugs in STS play an important role in identifying patients avoiding adverse events, and optimizing drug dose. The aim of these investigations is to take advantage of personalized chemotherapies regarding cancer treatment and prevention. Development in NGS technologies has been open a new opportunity for characterizing the genomic landscape of these tumors, together with the possibility of applying the genetic diagnostic tests relevant in cost-benefit analysis. However, due to the several rare STS histological subtypes harboring specific fusion genes (56), certain limitation should be considered for the most of the studies on NGS analyses that consider together samples from different STS histotypes where panel with a limited number of covered genes are used. In this particular point of view, the implementation of a panel containing an increased number of genes seems to be mandatory for a better daily diagnostic routine in STS.

Finally, future studies in this field should be considered in terms of identification and validation of drug-sensitivity test systems for routine use that include known specific PGx markers in common clinical management.

AUTHOR CONTRIBUTIONS

CC contributed to references collection of the study. CG contributed to the writing of this manuscript. All authors approved the final version.

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Next-Generation Sequencing Approaches for the Identification of Pathognomonic Fusion Transcripts in Sarcomas: The Experience of the Italian ACC Sarcoma Working Group

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This work describes the set-up of a shared platform among the laboratories of the Alleanza Contro il Cancro (ACC) Italian Research Network for the identification of fusion transcripts in sarcomas by using Next Generation Sequencing (NGS). Different NGS approaches, including anchored multiplex PCR and hybrid capture-based panels, were employed to profile a large set of sarcomas of different histotypes. The analysis confirmed the reliability of NGS RNA-based approaches in detecting sarcoma-specific rearrangements. Overall, the anchored multiplex PCR assay proved to be a fast and easy-to-analyze approach for routine diagnostics laboratories.

Keywords: sarcoma, molecular diagnosis, fusion transcripts, NGS, anchored multiplex PCR, hybrid capture-based panel

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INTRODUCTION

The term "sarcoma" identifies a heterogeneous group of rare tumors comprising over 60 different histologic variants (1). Due to their rarity and heterogeneity, the accuracy of sarcoma diagnosis remains challenging. In the diagnosis of sarcomas, tumor cell morphology (shape, pattern of growth, microenvironment contexture) and the expression of differentiation markers

represent the most important factors, but molecular investigations are increasingly employed to complement these pathological assessments. Indeed, the identification of histotype-specific (pathognomonic) gene alterations is of paramount importance in the differential diagnosis among sarcoma variants, between malignant and benign mimics, as well as between sarcoma and other tumor types (1–3). In particular, about one third of all sarcomas presents pathognomonic chromosome rearrangements (translocations, deletions, insertions) that result in fusion genes and corresponding expression of fusion transcripts (4). Beside diagnostic relevance, the expression of fusion transcripts may have prognostic and/or predictive implications. For example, certain rearrangements, such as those involving *ALK* in inflammatory myofibroblastic tumors or *COL1A1-PDGFB* in dermatofibrosarcoma protuberans, are predictive of the response to tyrosine kinase inhibitors (5, 6). Moreover, the detection of NTRK fusions in a broad range of malignancies, including sarcomas, has gaining much attention due to the recent demonstration of therapeutic efficacy of a new class of tyrosine kinase inhibitors in NTRK rearranged tumors (7–9).

Commonly, FISH or RT-PCR are used to detect fusion events at the genomic or transcriptional level, respectively. However, both methods present limitations. In particular, since they are suited to investigate a specific pre-defined abnormality, they inevitably rely on a prior diagnostic hypothesis (reflex testing). The advent of technologies such as next generation sequencing (NGS), aka massive parallel sequencing, has laid down the bases to overcome this limitation. By allowing the simultaneous analysis of a large set of targets (from few genes to the whole transcriptome/genome) NGS has disclosed the possibility not only to reveal diagnostic/prognostic/predictive genetic abnormalities in the absence of a prior hypothesis but also to identify new aberrations (10–12).

Here we wanted to assess feasibility, reliability, and applicability of NGS-based methods for the detection of sarcoma-associated fusion transcripts in a routine diagnostic setting. Our multicentric analysis confirms the sensitivity of anchored-based NGS profiling approaches and corroborates the suitability of these investigations in the diagnostic setting of sarcomas.

MATERIALS AND METHODS

Case Selection

The study was conducted on a series of 150 sarcoma samples, representative of different sarcoma histotypes, retrieved from the pathological files of the participating institutions (Alleanza Contro il Cancro, ACC, Italian Research Network). Either Formalin-Fixed Paraffin-Embedded (FFPE) or frozen samples were analyzed. All sarcomas included in the study were histopathologically re-evaluated on hematoxylin-eosin stained slides, and representative areas were selected for molecular analyses.

NGS-based Fusion Transcript Identification

RNA was extracted from 5 to 10 μm-FFPE tissue sections using the Qiagen miRNeasy FFPE kit (Qiagen, Valencia, CA, USA) or the Invitrogen RecoverAll Total Nucleic Acid Isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). For frozen samples the TRIzol reagent (Life Technologies Italia, Monza, Italy) followed by the RNeasy MinElute cleanup (Qiagen, Valencia, CA, USA) was used. Total RNA was quantified by using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Quality was checked with the RNA 6000 Nano Kit on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), or by using the Archer PreSeq^{TM} RNA QC qPCR Assay (ArcherDX, Boulder, CO, USA) and a threshold of DV_{200} >30 or PreSeq Cq <31 was used to identify high quality RNA, respectively.

FISH, RT-PCR, RT-qPCR, and IHC, used as primary detection approaches for the detection of possible fusion events, were performed during routine diagnostic procedures according to laboratory standard guidelines and validated reagents.

Three different commercially available NGS-based fusion panels were selected based on their capacity to cover most genes known to be involved in sarcoma-relevant fusions: an anchored multiplex PCR-based assay, namely the Archer FusionPlex Sarcoma kit (AMP-FPS)(ArcherDX, Boulder, CO, USA), covering 26 genes involved in sarcoma-associated fusions; two hybrid capture-based (HC) assays, namely the TruSight RNA Fusion Panel (TS-Fusion) (Illumina Inc., San Diego, CA, USA) and the TruSight RNA PanCancer Panel (TS-PanCancer) (Illumina Inc., San Diego, CA, USA) covering 507 and 1,385 genes commonly involved in cancer, respectively. Both HC assays included the 26 genes covered by the AMP-FPS kit. In a subset of samples, a customized version of the AMP-FPS panel was used to detect PAX3 fusion transcripts. Specifically, the assay was integrated with PAX3-specific primers (exons 6, 7 and 8) designed by using the Archer Assay Designer tool (ArcherDX, Boulder, CO, USA).

Libraries for all three panels were prepared and checked for quality according to the manufacturer's instructions, starting from 100 to 250 ng of RNA as input.

AMP-FPS libraries were run on either Illumina (MiSeq or NextSeq 500 Illumina Inc., San Diego, CA, USA) or Thermo (Ion S5 Thermo Fisher Scientific, Waltham, MA, USA) sequencing platforms, according to the manufacturer's instructions. HC-based libraries were sequenced on Illumina MiSeq instruments. Illumina TS-Fusion and TS-PanCancer sequencing data were analyzed by using the dedicated Illumina BaseSpace RNA-Seq Alignment tool (v.s.2.0.2), which relies on STAR and Manta algorithms (13, 14). PAR-masked/(RefSeq)hg19 was used as reference genome. A minimum of 3 million reads was obtained per sample (range 3007307–6284475). The mean percentage of reads aligned to the human genome was 98.9% (range 96.4–99.7%); the mean proportion of reads aligned to ribosomal RNA was below 2% (range 0.2–6.1%) and mean insert size was 134 bp

Abbreviations: NGS, next generation sequencing; FFPE, Formalin-Fixed Paraffin-Embedded; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcriptase-PCR; RT-qPCR, reverse transcriptase-quantitative PCR; IHC, immunohistochemistry; HC, hybrid capture-based panel; AMP-FPS, Anchored Multiplex PCR FusionPlex Sarcoma panel; TS-Fusion, TruSight RNA Fusion panel; TS-PanCancer, TruSight RNA PanCancer panel

(range 107–155 bp), in line with literature data (15). Only high-confidence fusions that passed default thresholds of the RNA-Seq Alignment tool (PASS) were recorded.

The Archer Analysis suite (v 5.1 or v 6.0) was exploited for the analysis of AMP-FPS panel results, using default settings. Default parameters (QC PASS) that, according to the Archer user manual, allow to achieve up to 95% of sensitivity in fusion detection, were employed to assess data quality. Samples included in the study met the quality cutoffs set by the Archer Analysis platform but in a few cases that, although not fulfilling all default criteria, nevertheless yielded high confidence fusion calls (cases #9, 31, 37, 47, 57, 60, 80, 126). Fusions were recorded as "high confidence calls"(strong = true in output table) if they passed all "strong evidence" default filters as described in the Archer analysis user manual (briefly: breakpoint spanning reads that support the candidate $\geq$ 5; "fusion_percent_of_GSP2_reads", i.e., proportion of breakpoint spanning reads that support the candidate relative to the total number of reads spanning the breakpoint $\geq$10%; "min_unique_start_sites_for_strong_fusion" $\geq$3; fusion recorded in the Quiver database or not fulfilling the "negative evidence criteria").

Of 48 cases (12 of the first set and 36 of the second set) where a fusion was detected by NGS but the partner genes had not been previously determined by the primary detection method, material was available for orthogonal validations (RT-PCR) in 39 cases, confirming NGS results. The involvement of *SSX4 (SS18-SSX4),* called sometime by the AMP-FPS assay in synovial sarcoma samples, was checked by nested RT-PCR (primers: Fw-SS18 GGACCACCACAGCCACCCCA, Rev-SSX ATGTTTCCCCCTTTTGGGTC; Rev-SSX4 GTCTTGTTAATC TTCTCCAAGG) and Sanger sequencing on a single index case.

For second level bioinformatic analyses of HC library raw data, Arriba, STAR-Fusion and Pizzly (16–18), administered through a command line interface, were employed for fusion calling using default settings.

RESULTS

NGS-based Identification of Fusion Transcripts: Panel Comparison

As a first step toward the assessment of suitability of NGS-based approaches for the detection of pathognomonic fusions in sarcomas, performance and ease-of-use (library preparation complexity, hands-on time, user-friendly dedicated bioinformatic analysis tool) of three different NGS fusion panels were evaluated on a set of sarcoma samples previously characterized by either FISH or RT-qPCR for gene fusions (**Table 1**). Twenty-six samples were analyzed with a hybrid capture-based panel (HC) (Illumina TS-Fusion). Twenty samples were analyzed with an anchored multiplex PCR panel (Archer AMP-FPS), 19 of which investigated also with the Illumina TS-Fusion. In addition, 9 samples were profiled with a more comprehensive HC panel (Illumina TS-PanCancer).

All three targeted RNA-sequencing panels permit the identification of common and known fusions involved in sarcomas, but also the discovery of novel fusions. The AMP-FPS panel targets a limited set of genes (26 target genes) that are commonly involved in sarcoma-associated fusions. This AMP-FPS panel employs unidirectional gene-specific primers to detect fusion transcripts involving target genes. In addition, molecular barcodes are included to enable single molecule counting, de-duplication and error correction, thus allowing quantitative analysis and confident mutation calling.

In HC-based panels the transcripts of interest are enriched by hybridization and capture with biotinylated probes (507 genes in TS-Fusion, 1385 genes in TS-PanCancer, in both cases including the 26 genes targeted by the AMP-FPS panel).

Raw data obtained with the different panels were then analyzed using the dedicated bioinformatic suite (BaseSpace RNA-Seq Alignment for Illumina HC panels, Archer Analysis platform for the AMP-FPS panel). The AMP-FPS assay correctly identified the pathognomonic fusion in all samples analyzed (20/20), irrespective of the sequencing platform used (Thermo and/or Illumina), demonstrating an excellent sensitivity. The pathognomonic fusion was correctly called in 22/26 samples analyzed with the TS-Fusion HC assay. Of the 9 cases analyzed with the TS-PanCancer HC panel, the dedicated bioinformatic tool identified the diagnostic fusion in 7 cases, in one of these as a reciprocal fusion. To further explore the performance of HC panels, data generated with TS-Fusion and TS-PanCancer panels were re-evaluated with additional algorithms, namely Arriba, STAR-Fusion and Pizzly (16–18). Although impractical in a routine diagnostic setting, as they rely on a command line interface, these tools are reported to have high fusion detection rates (16–18). With the exception of case #27, for which no algorithm detected, as high confidence calls, fusions involving the *CIC* gene, apparently rearranged according to FISH, at least one fusion caller was capable of detecting, among others, a fusion transcript involving the target gene in cases previously scored negative with the BaseSpace RNA-Seq Alignment tool, emphasizing the importance of software sensitivity in data analysis (**Supplemental Tables 1–3**).

Additional passing filters fusions (in frame and out of frame) were occasionally called beside the pathognomonic one, but the actual biological significance of these alterations is unclear. For instance, beside the canonical fusion involving *SS18* and *SSX1* or *SSX2,* additional fusions involving *SSX4* were called in 5/6 synovial sarcomas analyzed with the AMP-FPS panel. It should be pointed out that the AMP-FPS approach relies on relatively small amplicons. Thus, in the presence of highly homologous genes (e.g., *SSX1*, *SSX2*, *SSX4*), this technique may fail to properly distinguish the target (19). Indeed, a deeper analysis of an index case confirmed the expression of *SS18-SSX1,* suggesting that the alleged *SS18-SSX4* fusion was likely an alignment artifact.

Overall, both AMP-FPS and HC assays demonstrated a good detection capability. The HC assays were definitively more comprehensive and suitable for a research environment. In contrast, the AMP-FPS panel was limited in breath (only 26 target genes), and hence with reduced capacity of discovering new fusions, but definitively provided for a better ease-of-use. In particular, the hands-on-time for library preparation was reduced. Moreover, compared to the BaseSpace RNA-Seq Alignment, the AMP-FPS dedicated bioinformatic analysis tool (Archer Analysis platform) featured a more user-friendly graphical interface with detailed and straightforward information

TABLE 1 | NGS fusion profiling: panel comparison.

Nr	Diagnosis	Pre-detected genetic abnormality	Primary detection method	Histotype-specific fusion detected by the indicated NGS approach			Other passing filters fusions (assay detecting the additional fusion)
				AMP-FPS	**TS-Fusion**	**TS-PanCancer**	
1	Dermatofibrosarcoma Protuberans	*PDGFB*	FISH	*COL1A1-PDGFB*^{IL}	*COL1A1-PDGFB*	*COL1A1-PDGFB*	NFD
2	Ewing Sarcoma	*EWSR1*	FISH	*EWSR1-FLI1*^{IL}	*EWSR1-FLI1*	*EWSR1-FLI1*	NFD
3	Infantile Fibrosarcoma	*ETV6*	FISH	*ETV6-NTRK3*^{IL}	*ETV6-NTRK3*	*ETV6-NTRK3*	NFD
4	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	*SS18-SSX1*^{IL}	*SS18-SSX1*	*SS18-SSX1*	*SS18-SSX4* (AMP-FPS^{IL})
5	Synovial Sarcoma	*SS18*	FISH	*SS18-SSX2*^{IL}	*SS18-SSX2*	*SS18-SSX2*	*SS18-SSX4* (AMP-FPS^{IL})
6	Myoepithelioma (soft tissue)	*EWSR1*	FISH	*EWSR1-ATF1*^{IL}	*EWSR1-ATF1*	NFD	*ATF1-EWSR1* (TS-Fusion,TS-PanCancer)
7	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	*EWSR1-NR4A3*^{IL}	NFD	NFD	NFD
8	Clear Cell sarcoma	*EWSR1*	FISH	*EWSR1-ATF1*^{T,IL}	NFD	nd	NFD
9	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	*EWSR1-FLI1*^{T,IL}	*EWSR1-FLI1*	nd	NFD
10	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	*EWSR1-FLI1*^{T,IL}	*EWSR1-FLI1*	nd	NFD
11	Ewing Sarcoma	*EWSR1-ERG*	RT-qPCR	*EWSR1-ERG*^{T,IL}	*EWSR1-ERG*	nd	*EWSR1-ERG-EWSR1* (AMP-FPS^{IL})
12	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	*EWSR1-NR4A3*^{T}	*EWSR1-NR4A3*	nd	NFD
13	Myxoid Liposarcoma	*FUS-DDIT3*	RT-qPCR	*FUS-DDIT3*^{IL}	*FUS-DDIT3*	nd	NFD
14	Myxoid Liposarcoma	*FUS-DDIT3*	RT-qPCR	*FUS-DDIT3*^{T,IL}	*FUS-DDIT3*	nd	*DDIT3-FUS* (TS-Fusion)
15	Myxoid Liposarcoma	*FUS-DDIT3*	RT-qPCR	*FUS-DDIT3*^{T,IL}	*FUS-DDIT3*	nd	*FUS-DDIT3-DLG2* (AMP-FPS^{IL})
16	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	*SS18-SSX1*^{IL}	*SS18-SSX1*	nd	*SS18-SSX4-SS18; SS18-SSX4* (AMP-FPS^{IL})
17	Synovial Sarcoma	*SS18*	FISH	*SS18-SSX1*^{IL}	*SS18-SSX1*	nd	NFD
18	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	*SS18-SSX1*^{IL}	*SS18-SSX1*	nd	*SS18-SSX4* (AMP-FPS^{IL})
19	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	*SS18-SSX1*^{T,IL}	*SS18-SSX1*	nd	*SS18-SSX1/4-SS18; SS18-SSX4* (AMP-FPS^{IL})
20	Myxoid Liposarcoma	*DDIT3*	FISH	*FUS-DDIT3*^{IL}	nd	*FUS-DDIT3*	*DDIT3-FUS* (TS-PanCancer)
21	Myxoid Liposarcoma	*DDIT3*	FISH	nd	FUS-DDIT3	NFD	NFD
22	Synovial Sarcoma	*SS18*	FISH	nd	*SS18-SSX1*	nd	NFD
23	Synovial Sarcoma	*SS18*	FISH	nd	*SS18-SSX1*	nd	NFD
24	Myxoid Fibrosarcoma	*FUS*	FISH	nd	*FUS-CREB3L2*	nd	NFD
25	Myxoid Liposarcoma	*FUS-DDIT3*	RT-qPCR	nd	*FUS-DDIT3*	nd	*DDIT3-FUS* (TS-Fusion)
26	Myxoid Liposarcoma	*DDIT3*	FISH	nd	NFD	nd	NFD
27	Undifferentiated Round Cell, Ewing-Like Sarcoma	*CIC*	FISH	nd	NFD	nd	NFD

NFD, no histotype-specific fusion detected; nd, not done; FISH, fluorescent in situ hybridization; RT-qPCR, reverse transcriptase- quantitative PCR; Sequencing platform used: T, Thermo platform; IL, Illumina platform.

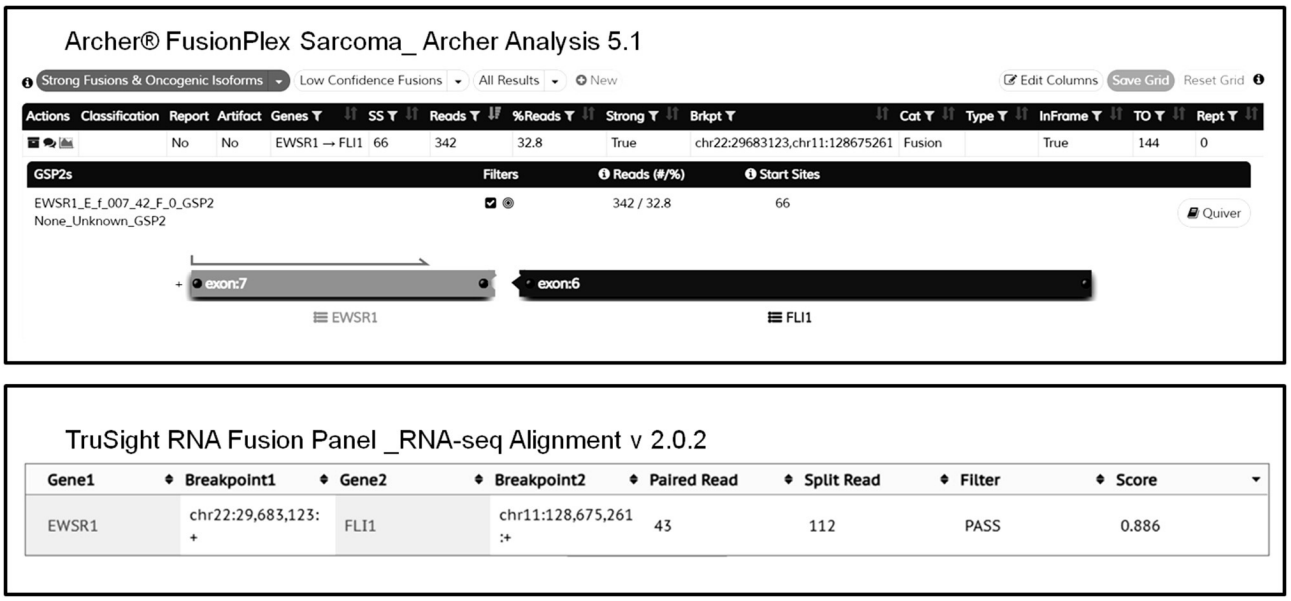

FIGURE 1 | Representative graphical output of Archer Analysis (top) and Illumina BaseSpace RNA-Seq Alignment (bottom) tools. The EWSR1-FLI1 fusion detected in sample #2 by both AMP-FPS and HC panels is shown.

about the fusion (exons involved, in frame/out of frame, confidence of the call) (**Figure 1**).

On the whole, we considered the AMP-FPS assay more suitable for routine diagnostics.

Validation on a Larger Set of Cases of the AMP-FPS Fusion Transcript Assay

Based on these results, with a view to translating NGS-based fusion identification in a routine diagnostic setting, we sought to extend the evaluation of the AMP-FPS panel (on either a Thermo or an Illumina sequencing platform) to 123 additional cases (**Table 2**).

Overall, the AMP-FPS panel confirmed the good performance. Of 81 cases with a pre-detected genetic abnormality suggestive of a fusion event, this NGS assay proved effective in 71, with orthogonal validations (RT-PCR) confirming the NGS result where appropriate (see Material and Methods). In the remaining 10 cases, a gene rearrangement was suggested by FISH. Nevertheless, although samples passed quality filters, the AMP-FPS assay failed to detect a fusion transcript. There are several possible explanations for this discrepancy including inadequate tumor cell fraction or low expression levels of the fusion transcript, chromosome rearrangements not yielding a fusion transcript, unusual breakpoints not covered by the assay or lack of primers covering the target gene. For instance, in two tumors (one endometrial stromal sarcoma and one sarcoma NOS) FISH indicated a rearrangement of the *BCOR* gene with an unknown partner. It is worth noting that the commercial AMP-FPS panel used in this study does not include primers for *BCOR*. Moreover, beside the common *CCNB3* partner (covered by the panel), *BCOR* has been reported to fuse with other genes which are also not targeted by the AMP-FPS assay (e.g., *ZC3H7B*, *MAML3*, *CIITA*) (20–23). Thus, in the absence of probes for *BCOR* and potential partner genes, the failure of the assay in the 2 *BCOR* rearranged tumors of our series is not surprising. The same holds true for rearrangements involving *NR4A3* in extraskeletal myxoid chondrosarcomas: while the AMP-FPS assay covers the most *NR4A3* common partners (*EWSR1, TAF15, TCF12, TFG*) it lacks probes for both *NR4A3* and uncommon partners (24), thus scoring negative in the presence of alternative fusions.

The AMP-FPS assay failed to detect any fusion also in 3 cases of biphenotypic sinonasal sarcoma. Although in these cases no prior investigation (FISH or RT-PCR) was performed, this tumor is known to be typified by gene fusions involving the *PAX3* gene (25). Since the *PAX3* gene is not covered by the commercial AMP-FPS panel, we commissioned a customization of the assay by spiking-in primers to cover *PAX3* fusions. By using this customized AMP-FPS assay we were able to demonstrate and validate that all 3 cases expressed a *PAX3-MAML3* chimeric transcript (**Figure 2**).

Interestingly, a rare EWSR1-PATZ1 fusion was detected by AMP-FPS in one EWSR1 FISH-positive Ewing sarcoma (case #34). This fusion had been previously described in rare cases of spindled or small round cell sarcomas and it is considered to identify a distinct, Ewing-like entity (26). Moreover, the NGS profiling allowed the detection of disease-associated fusion transcripts also in a set of cases for which no prior molecular data was available or scored negative for FISH. These included one dermatofibrosarcoma protuberans (*COL1A1-PDGFB*), one endometrial stromal sarcoma (*YWHAE-NUTM2B*, aka *YWHAE-FAM22B*), one gastrointestinal neuroectodermal tumor (*EWSR1-CREB1*), one inflammatory myofibroblastic sarcoma (*TPM4-ALK*), one inflammatory myofibroblastic tumor (*TFG-ROS1*), 2 myoepitheliomas (one *FUS-NFATC2* and one *TRPS1-PLAG1*), 2 sclerosing epithelioid fibrosarcomas (one *EWSR1-CREB3L2* and one *FUS-CREB3L2*) and one solitary

TABLE 2 | Validation of the AMP-FPS fusion transcript assay.

Nr	Diagnosis	Pre-detected genetic abnormality	Primary detection method	Sequencing platfom	Histotype-specific fusion detected	Other passing filters fusions
28	Askin Tumor	*EWSR1-ERG*	RT-qPCR	Illumina	*EWSR1-ERG*	*EWSR1-unl-ERG*
29	Congenital Fibrosarcoma	*ETV6-NTRK3*	RT-qPCR	Illumina	*ETV6-NTRK3*	NFD
30	Dermatofibrosarcoma Protuberans	*COL1A1-PDGFB*	FISH	Thermo	*COL1A1-PDGFB*	NFD
31	Dermatofibrosarcoma Protuberans	*COL1A1-PDGFB*	RT-qPCR	Illumina	*COL1A1-PDGFB*	NFD
32	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR-FLI1*	NFD
33	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR-FLI1*	NFD
34	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR1-PATZ1*	NFD
35	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR-FLI1*	NFD
36	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR-FLI1*	NFD
37	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Illumina	*EWSR1-FLI1*	*FXR2-CAMTA1*
38	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Illumina	*EWSR1-FLI1*	NFD
39	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Illumina	*EWSR1-FLI1*	NFD
40	Ewing Sarcoma	*EWSR1-ERG*	RT-qPCR	Illumina	*EWSR1-ERG*	*EWSR1-unl-EWSR1-ERG; FUS-ERG; EWSR1-ERG-EWSR1;*
41	Ewing Sarcoma	*EWSR1-FLI1*	FISH	Illumina	*EWSR1-FLI1*	*EWSR1-FLI1-EWSR1*
42	Ewing Sarcoma	*EWSR1*	FISH	Thermo	*EWSR1-FLI1*	NFD
43	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
44	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
45	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
46	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
47	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
48	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
49	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Thermo	*EWSR1-FLI1*	NFD
50	Ewing Sarcoma	*EWSR1-FLI1*	RT-qPCR	Illumina	*EWSR1-FLI1*	NFD
51	Ewing Sarcoma	*EWSR1*	FISH	Illumina	*EWSR1-FLI1*	NFD
52	Ewing Sarcoma	*FUS*	FISH	Thermo	*FUS-ERG*	NFD
53	Ewing-like Sarcoma	*BCOR-CCNB3*	RT-qPCR	Illumina	*BCOR-CCNB3*	NFD
54	Ewing-like Sarcoma	*CIC-DUX4*	RT-qPCR	Illumina	*CIC-DUX4*	NFD
55	Extraskeletal Myxoid Chondrosarcoma	*NR4A3*	FISH	Illumina	*EWSR1-NR4A3*	NFD
56	Extraskeletal Myxoid Chondrosarcoma	*EWSR1*	FISH	Illumina	*EWSR1-NR4A3*	NFD
57	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
58	Extraskeletal Myxoid Chondrosarcoma	*TAF15-NR4A3*	RT-qPCR	Illumina	*TAF15-NR4A3*	NFD
59	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
60	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
61	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
62	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
63	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD
64	Extraskeletal Myxoid Chondrosarcoma	*NR4A3*	FISH	Illumina	*EWSR1-NR4A3*	NFD
65	Extraskeletal Myxoid Chondrosarcoma	*EWSR1-NR4A3*	RT-qPCR	Illumina	*EWSR1-NR4A3*	NFD

(Continued)

TABLE 2 | Continued

Nr	Diagnosis	Pre-detected genetic abnormality	Primary detection method	Sequencing platfom	Histotype-specific fusion detected	Other passing filters fusions
66	Myoepitelial carcinoma (soft tissue)	*EWSR1*	FISH	Illumina	*EWSR1-ATF1*	NFD
67	Myoepithelioma (soft tissue)	*EWSR1*	FISH	Illumina	*EWSR1-ATF1*	NFD
68	Myxoid Liposarcoma	*FUS-DDIT3*	RT-PCR	Thermo	*FUS-DDIT3*	NFD
69	Myxoid Liposarcoma	*FUS-DDIT3*	RT-qPCR	Illumina	*FUS-DDIT3*	NFD
70	Myxoid Liposarcoma	*FUS-DDIT3*	FISH	Thermo	*FUS-DDIT3*	NFD
71	Myxoid Liposarcoma	*FUS-DDIT3*	FISH	Illumina	*FUS-DDIT3*	NFD
72	Myxoid Liposarcoma	*FUS-DDIT3*	FISH	Illumina	*FUS-DDIT3*	NFD
73	Nodular Fascitis	*USP6*	FISH	Thermo	*MYH9-USP6*	NFD
74	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-PCR	Thermo	*PAX3-FOXO1*	NFD
75	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-PCR	Thermo	*PAX3-FOXO1*	NFD
76	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-PCR	Thermo	*PAX3-FOXO1*	NFD
77	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3-FOXO1*	NFD
78	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3-FOXO1*	NFD
79	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3-FOXO1*	NFD
80	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3-FOXO1*	NFD
81	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3 - FOXO1*	*FOXO1-PAX3*
82	Rhabdomyosarcoma, alveolar	*PAX3-FOXO1*	RT-qPCR	Illumina	*PAX3-FOXO1*	NFD
83	Rhabdomyosarcoma, splindle cell	*SRF-NCOA2*	RT-qPCR	Illumina	*SRF- NCOA2*	NFD
84	Sarcoma NOS	*EWSR1*	FISH	Illumina	*EWSR1-FLI1*	NFD
85	Solitary Fibrous Tumor	*STAT6*	IHC	Thermo	*NAB2-STAT6*	NFD
86	Synovial Sarcoma	*SS18-SSX2*	RT-qPCR	Illumina	*SS18-SSX2*	*SS18-SSX4;SS18-SSX1; complex SS18-SSX2 fusions*
87	Synovial Sarcoma	*SS18*	FISH	Illumina	*SS18-SSX1*	*SS18-SSX4; SS18-SSX4-SS18*
88	Synovial Sarcoma	*SS18*	FISH	Thermo	*SS18-SSX1*	NFD
89	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Illumina	*SS18-SSX1*	NFD
90	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Thermo	*SS18-SSX1*	NFD
91	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Thermo	*SS18-SSX1*	*SS18-SSX2*
92	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Thermo	*SS18-SSX1*	*SS18-SSX4*
93	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Thermo	*SS18-SSX1*	*SS18-SSX4*
94	Synovial Sarcoma	*SS18*	FISH	Illumina	*SS18-SSX1*	*SS18-SSX4-SS18*
95	Synovial Sarcoma	*SS18-SSX2*	RT-qPCR	Illumina	*SS18-SSX2*	NFD
96	Synovial Sarcoma	*SS18*	FISH	Illumina	*SS18-SSX1*	*SS18-SSX4*
97	Synovial Sarcoma	*SS18-SSX1*	RT-qPCR	Thermo	*SS18-SSX1*	*SS18-SSX4*
98	Clear Cell Sarcoma	*EWSR1*	FISH	Thermo	*EWSR1-CREB1*	NFD
99	Endometrial Stromal Sarcoma	*BCOR*	FISH	Thermo	NFD	NFD
100	Extraskeletal Myxoid Chondrosarcoma	*NR4A3*	FISH	Illumina	NFD	NFD
101	Myoepithelioma (soft tissue)	*EWSR1*	FISH	Illumina	NFD	NFD
102	Myxoid Fibrosarcoma	*FUS*	FISH	Illumina	NFD	NFD

(Continued)

TABLE 2 | Continued

Nr	Diagnosis	Pre-detected genetic abnormality	Primary detection method	Sequencing platfom	Histotype-specific fusion detected	Other passing filters fusions
103	Myxoid Liposarcoma	*DDIT3*	FISH	Illumina	NFD	NFD
104	Nodular Fasciitis	*USP6*	FISH	Thermo	NFD	NFD
105	Rhabdomyosarcoma, alveolar	*FOXO1*	FISH	Thermo	NFD	NFD
106	Sarcoma NOS	*BCOR*	FISH	Thermo	NFD	NFD
107	Solitary Fibrous Tumor	*EWSR1*	FISH	Illumina	NFD	NFD
108	Undifferentiated round cell, Ewing-Like Sarcoma	*CIC*	FISH	Illumina	NFD	NFD
109	Lipoblastoma	*PLAG1 neg*	FISH	Illumina	NFD	NFD
110	Myxoid Fibrosarcoma	*EWSR1, FUS neg*	FISH	Thermo	NFD	NFD
111	Myxoid Fibrosarcoma	*EWSR1, FUS neg*	FISH	Thermo	NFD	NFD
112	Myxoid Fibrosarcoma	12q13-15 amp	FISH	Thermo	NFD	NFD
113	Rhabdomyosarcoma, alveolar	*FOXO1 neg*	FISH	Thermo	NFD	NFD
114	Rhabdomyosarcoma, embryonal	*FOXO1 neg*	FISH	Illumina	NFD	NFD
115	Rhabdomyosarcoma, embryonal	*FOXO1 neg*	FISH	Illumina	NFD	NFD
116	Rhabdomyosarcoma, embryonal	*FOXO1 neg*	FISH	Illumina	NFD	NFD
117	Sarcoma NOS	*EWSR1 neg*	FISH	Illumina	*CIC-DUX4*	NFD
118	Small Round Cell Tumor	*EWSR1, BCOR, FUS, CIC neg*	FISH	Thermo	NFD	NFD
119	Undifferentiated Sarcoma	*EWSR1 neg*	FISH	Illumina	*CIC-DUX4*	NFD
120	Undifferentiated Sarcoma	12q13-15 amp	FISH	Thermo	NFD	NFD
121	Undifferentiated Sarcoma	12q13-15 amp	FISH	Thermo	NFD	*HMGA2-LGR5*
122	Biphenotypic Sinonasal Sarcoma	nd	nd	Thermo	*PAX3-MAML3*§	NFD
123	Biphenotypic Sinonasal Sarcoma	nd	nd	Thermo	*PAX3-MAML3*§	NFD
124	Biphenotypic Sinonasal Sarcoma	nd	nd	Thermo	*PAX3-MAML3*§	NFD
125	Dermatofibrosarcoma Protuberans	nd	nd	Thermo	*COL1A1-PDGFB*	NFD
126	Endometrial Stromal Sarcoma	nd	nd	Thermo	*YWHAE-NUTM2B*	NFD
127	Gastrointestinal Neuroectodermal Tumor	nd	nd	Thermo	*EWSR1-CREB1*	*SS18-PTRF*
128	Inflammatory Myofibroblastic Sarcoma	nd	nd	Illumina	*TPM4-ALK*	NFD
129	Inflammatory Myofibroblastic Tumor	nd	nd	Thermo	*TFG-ROS1*	NFD
130	Myoepithelioma (bone)	nd	nd	Illumina	*FUS-NFATC2*	NFD
131	Myoepithelioma (soft tissue)	nd	nd	Illumina	*TRPS1-PLAG1*	NFD
132	Sclerosing Epitheliodid Fibrosarcoma	nd	nd	Illumina	*EWSR1-CREB3L2*	NFD
133	Sclerosing epitheliodid fibrosarcoma (soft tissue)	nd	nd	Illumina	*FUS-CREB3L2*	NFD
134	Solitary Fibrous Tumor	nd	nd	Thermo	*NAB2-STAT6*	NFD
135	Chondrosarcoma	nd	nd	Thermo	NFD	NFD
136	Endometrial Stromal Sarcoma	nd	nd	Thermo	NFD	NFD
137	Epithelioid Angiosarcoma	nd	nd	Illumina	NFD	NFD

(Continued)

TABLE 2 | Continued

Nr	Diagnosis	Pre-detected genetic abnormality	Primary detection method	Sequencing platfom	Histotype-specific fusion detected	Other passing filters fusions
138	Follicular Dendritic Cell Sarcoma	nd	nd	Thermo	NFD	NFD
139	Leiomyosarcoma	nd	nd	Illumina	NFD	NFD
140	Leiomyosarcoma	nd	nd	Thermo	NFD	NFD
141	Myoepithelioma (bone)	nd	nd	Illumina	NFD	NFD
142	Myxoid Fibrosarcoma	nd	nd	Thermo	NFD	NFD
143	Myxoinflammatory Fibroblastic Sarcoma	nd	nd	Illumina	NFD	NFD
144	Osteosarcoma	nd	nd	Illumina	NFD	NFD
145	Osteosarcoma	nd	nd	Illumina	NFD	NFD
146	Pleomophic Sarcoma	nd	nd	Thermo	NFD	NFD
147	Pleomophic Sarcoma	nd	nd	Thermo	NFD	NFD
148	Pleomophic Sarcoma	nd	nd	Thermo	NFD	NFD
149	Sarcoma NOS HG Myxoid	nd	FISH	Thermo	NFD	NFD
150	Undifferentiated Sarcoma	nd	nd	Illumina	NFD	NFD

NFD, no histotype-specific fusion detected; nd, not done; amp, amplification; neg, negative; RT-PCR, reverse transcriptase-PCR; FISH, fluorescent in situ hybridization; RT-qPCR, reverse transcriptase-quantitative PCR; IHC, immunohistochemistry; unl, unaligned sequence. PAX3-MAML3§: fusion detected with a PAX3-customized AMP-FPS Panel. This sample scored negative with the standard AMP-FPS Panel.

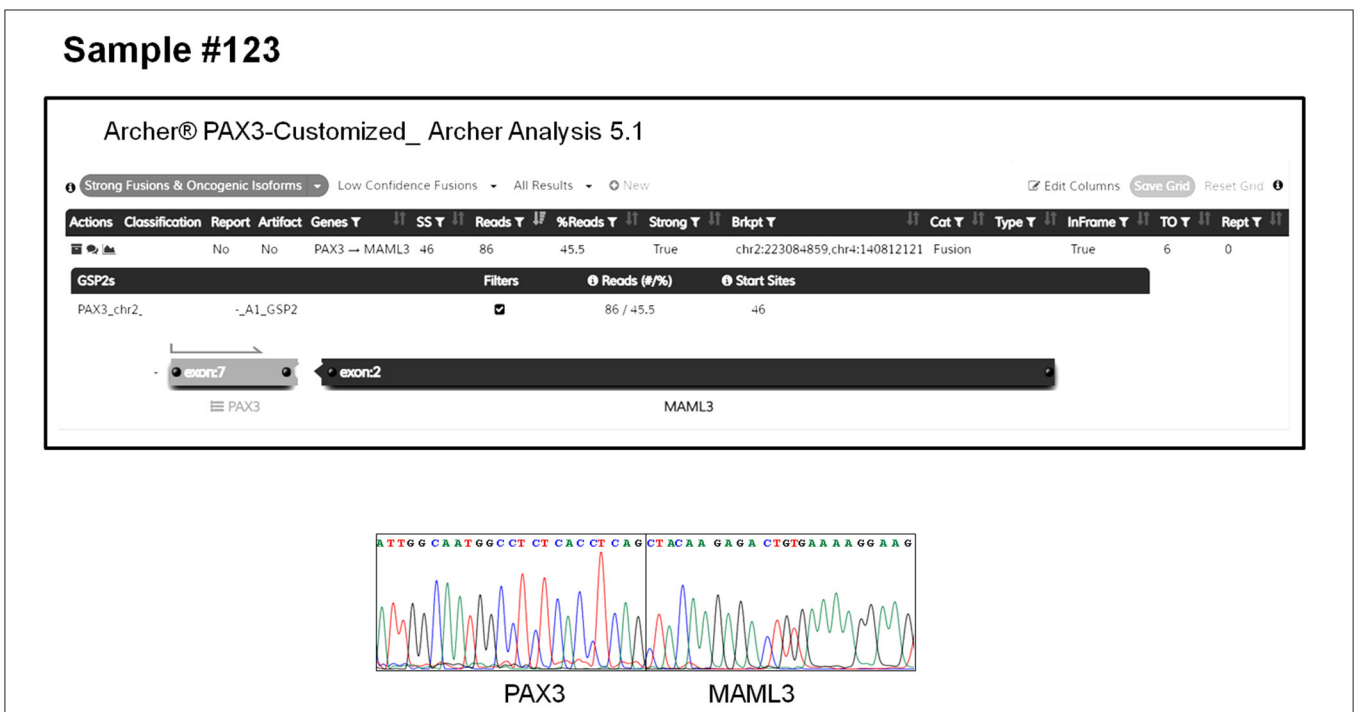

FIGURE 2 | PAX3-MAML3 fusion detected by the customized AMP-FPS panel in a representative case of biphenotypic sinonasal sarcoma (sample #123). The top panel shows the output of the Archer Analysis tool. The bottom panel shows the validation of the fusion by RT-PCR sequencing.

fibrous tumor (*NAB2-STAT6*). In addition, 2/5 tumors negative for *EWSR1* rearrangements according to FISH, turned out to express a *CIC-DUX4* fusion, leading to the diagnosis of *CIC-DUX4* fusion-positive undifferentiated round cell sarcoma (27). In all these cases the identified fusions were confirmed by RT-PCR.

Finally, the series analyzed included also sarcoma variants typically devoid of pathognomonic fusions (e.g., leiomyosarcoma, osteosarcoma). Thus, the negative result of the NGS profiling in these cases may be considered compatible with the pathological diagnosis.

DISCUSSION

The expression of fusion transcripts characterizes over a third of sarcomas where it may provide diagnostic, prognostic and predictive information. The cooperative effort described in this work was aimed at assessing feasibility, reliability, and applicability of NGS-based approaches for the detection of pathognomonic fusion transcripts in a routine diagnostic setting.

In line with recent reports (12, 19), our study corroborates the robustness of NGS, and in particular of AMP-FPS profiling, for the detection of clinically relevant fusions in sarcomas. On one hand, our analysis emphasizes the worth of implementing this type of approach in routine diagnostics. On the other hand, it underlines the importance of being aware of the actual detection capability of the panel used (genes covered by the assay) in relation to the specific tumor variant under investigation.

Our study demonstrates also the versatility of certain NGS fusion commercial panels to respond to specific diagnostic needs. In fact, the possibility of further implementing commercially available panels by spiking-in probes for genetic targets not included in the standard version of the assay allows to expand its detection capability. Indeed, beside *PAX3*, due to the recent therapeutic successes of NTRK fusions targeting drugs in solid tumors (7, 8), we are in the process of customizing the AMP-FPS panel by including primers for *NTRK1* and *NTRK2* (currently only *NTRK3* is covered by the AMP-FPS assay).

Importantly, in the presence of a negative result, a re-evaluation of RNA and library quality is mandatory as highly degraded RNA and poor quality libraries may affect the sensitivity of the assay. Nonetheless, we found that apparently low quality samples may still be effective for fusion detection. Indeed, a few cases included in this study (cases #9, 31, 37, 47, 57, 60, 80, 126), although not fulfilling all quality criteria, nevertheless yielded a correct fusion call. This indicates that this type of assay may work even in suboptimal conditions.

Finally, when reporting the result of this type of NGS analysis, especially if negative, a statement specifying the characteristics and the limits of the assay employed (type of NGS panel, number of target genes, website of the provider for the list of targeted fusions) and the actual performance of the test according to the manufacturer's standards (fulfillment of quality parameters) should always be included in the pathology report. It is worth reaffirming that the AMP-FPS assay is designed to target the most common breakpoint regions of the genes covered by the assay. Thus, unusual breakpoints may be source of "false negative" results. Moreover, when dealing with sarcoma variants expressing uncommon fusions, the presence of primers for the target genes should be verified prior to setting up the profiling because the lack of appropriate primers will yield a false negative result. The negativity in the AMP-FPS assay of the two *BCOR* rearranged tumors, included in this series, is instructive in this regard.

In the case of a positive result, beside the genes involved in the fusion, the inclusion in the pathology report of details about the fusion variant detected, including reading frame of the chimeric transcript (in frame/out of frame) and exons involved might be useful. This is of particular importance if the fusion protein is potentially actionable and the retention of specific domains in the chimeric protein is crucial for drug sensitivity, as in the case of NTRK fusions (7–9).

DATA AVAILABILITY STATEMENT

Sequencing data files are available in the NCBI-SRA (http://www.ncbi.nlm.nih.gov/sra) database under the accession number PRJNA608250.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic committee Istituto Ortopedico Rizzoli IRCCS, Regina Elena National Cancer Institute IRCCS, Bambino Gesù Children's Hospital IRCCS and by the proper institutional review boards of the CRO Aviano IRCCS National Cancer Institute, Veneto Institute of Oncology (IOV) IRCCS, University of Padua, Candiolo Cancer Institute FPO-IRCCS, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) Meldola IRCCS, Istituto Nazionale dei Tumori di Milano Fondazione IRCCS. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RM conceived the work on the behalf of the ACC sarcoma working group. All authors contributed to the generation of molecular profiling data. Each center involved in panel sequencing was responsible for generation, analyses and sharing of data. RF and RM coordinated the collection and integration of data. DR, MB, DB, FG, and BC were in charge of panel comparison. DR, MB, and DB were in charge of second-level bioinformatic analyses. RM and RF wrote the first draft of the manuscript with the support of DR and MB. All authors revised and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00489/full#supplementary-material

Supplemental Table 1 | Fusion transcripts called by the Arriba algorithm.

Supplemental Table 2 | Fusion transcripts called by the Pizzly algorithm.

Supplemental Table 3 | Fusion transcripts called by the STAR-Fusion algorithm.

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Targeted Deep Sequencing Uncovers Cryptic KIT Mutations in KIT/PDGFRA/SDH/RAS-P Wild-Type GIST

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Background: Gastrointestinal stromal tumors (GIST) are known to carry oncogenic KIT or PDGFRA mutations, or less commonly SDH or NF1 gene inactivation, with very rare cases harboring mutant BRAF or RAS alleles. Approximately 10% of GISTs are devoid of any of such mutations and are characterized by very limited therapeutic opportunities and poor response to standard treatments.

Methods: Twenty-six sporadic KIT/PDGFRA/SDH/RAS-pathway wild type GIST were profiled for the molecular status of genes frequently altered in GIST by a targeted next generation sequencing (NGS) approach. Molecular findings were validated by alternative amplicon-based targeted sequencing, immunohistochemistry, gene expression profiling and Sanger sequencing.

Results: Three patients harboring NF1 inactivating mutations were identified and excluded from further analysis. Intriguingly, five patients carried cryptic KIT alterations, mainly represented by low-allele-fraction mutations (12–16% allele ratio). These mutations were confirmed by another targeted NGS approaches and supported by CD117 immuno-staining, gene expression profiling, Sanger sequencing, with peak signals at the level of background noise, and by the patients' clinical course assessment.

Conclusion: This study indicates that ∼20% patients diagnosed with a KIT/PDGFRA/SDH/RAS-pathway wild-type GIST are *bona-fide* carriers of pathogenic KIT mutations, thus expected to be eligible for and responsive to the various therapeutic lines of TK-inhibitors in use for KIT/PDGFRA-mutant GIST. The centralization for a second level molecular analysis of GIST samples diagnosed as wild-type for KIT and PDGFRA is once again strongly recommended.

Keywords: gastrointestinal stromal tumor, GIST, KIT, quadruple-WT, deep sequencing

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors arising in the gastrointestinal tract (1). Nearly 85% of GISTs are characterized by mutually exclusive activating mutations in KIT or PDGFRA receptors (2, 3), that lead to constitutive ligand-independent activation of receptor signaling and account for their sensitivity to tyrosine kinase inhibitors (TKI) (4, 5). TKIs are the standard therapeutic approach for patients with unresectable tumor, ensuring a significant improvement in the clinical outcome of patients with advanced disease. Approximately 10–15% of all sporadic GIST cases are devoid of mutations in either gene, and have always been classified as KIT/PDGFRA wild-type GIST (6). This very heterogeneous category includes around 20–40% of cases that are succinate dehydrogenase complex (SDH)-deficient GIST, due to germline and/or somatic loss-of-function mutations in any of the four SDH subunits (A, B, C, or D) (7–9). Another subgroup of KIT/PDGFRA wild-type GIST with intact SDH complex, collectively defined as RAS-pathway (RAS-P)-mutant GIST, includes patients that either carry inactivating mutations in NF1 gene, often signaling an unrecognized NF1 syndromic condition (10, 11), or activating mutations in BRAF or more rarely a RAS gene (12, 13). Hence there is only half of the KIT/PDGFRA WT GIST that are recognized as either SDH-deficient or BRAF/RAS/NF1 mutated, but still the other half remains orphan of a driver oncogenic event and possibly of a specific target for therapy. Up to now, only private or hardly recurrent alterations have been identified in this GIST subgroup, such as ETV6-NTRK3, FGFR1, or FGF4 alterations, MAX, MEN1 (14–17), and still no conclusive result has been found on the actionable mutations for this subset of patients. Conversely, many studies point to a common gene expression profile (15, 18), despite the heterogeneity of the genetic analysis, suggesting that some shared signaling pathway should be evoked by different genetic alterations.

Hence, in this study we sought to investigate in depth, by a targeted NGS approach, the genetic status of the so-called KIT/PDGFRA/SDH/RAS-P wild-type GIST, to uncover putative alterations in frequently mutated genes that could be missed by conventional molecular diagnostic approaches.

METHODS

Patient Series

The series consisted of archival FFPE tissues from 26 GIST specimens negative for KIT/PDGFRA/BRAF/NRAS/KRAS with intact SDH complex, that are designated here as quadruple-WT for clarity. GIST diagnosis was done by expert pathologists based on morphology and CD117 expression. The study was approved by the local Institutional Ethical Committee and informed consent was provided by all living patients.

KIT, PDGFRA, BRAF, KRAS, and NRAS mutational status was assessed by Sanger sequencing both by the local diagnostic service and replicated and confirmed by our referral Molecular Diagnostic Unit. In KIT/PDGFRA/BRAF/KRAS/NRAS-negative cases, SDH deficiency was assessed by IHC for SDHB, followed by Sanger sequencing of the four SDH subunits.

Targeted Deep Sequencing

Areas with more than 90% of tumor cells were selected by an expert pathologist and dissected for nucleic acid extraction. DNA was extracted using QiAmp DNA micro Kit (QIAGEN) and quantified using picogreen dsDNA assay (Life Technologies). TruSeq Custom Amplicon (TSCA) low input sequencing panel, covering the entire coding region of NF1, SDHA, SDHB, SDHC, SDHD, and selected exons of KIT (exons 8, 9, 11, 13, 14, 17, 18), PDGFRA (exons 12,14,18), BRAF (exons 11 and 15), NRAS (exons 2 and 3), and KRAS (exons 2, 3 and 4), was designed with Design Studio software (Illumina). All KIT and PDGFRA exons target of primary or secondary mutations indicated in the most recent guidelines on GIST molecular diagnostics were included in this panel (19, 20). BRAF, NRAS, and KRAS recurrent hotspot mutations were covered. Since the DNA was extracted from Formalin-Fixed Paraffin-Embedded (FFPE) specimens, we employed a dual-strand TruSeq Custom Amplicon (TSCA) approach, that is able to discriminate reads produced from positive and negative strands of DNA to exclude artifacts derived from tissue fixation. The average amplicon length was of 175 bp. Thirty nanograms of DNA extracted from 26 FFPE GIST samples were used for library synthesis following TSCA Low-Input Dual Strand kit (Illumina) guidelines. Briefly, for each region of interest, two custom probes were hybridized and elongated copying target DNA. The two elongation products were then ligated and amplificated adding Illumina adaptes and sequencing primers Illumina adapters and sequencing primers.

Libraries were then quantified using Quant-IT Picogreen dsDNA reagent (Thermo Fisher Scientific), normalized to 4 nM and pooled. Ten picomolar (pM) of pooled libraries were sequenced on a Micro V2 flowcell on Miseq platform (Illumina) at 150 bp read length in paired-end mode, reaching an average depth of 295X.

To confirm the presence of low-allele-fraction mutations in the KIT gene, a complementary targeted sequencing approach was also employed, based on deep sequencing of PCR amplicons of target KIT exons. DNA library preparation was performed with Nextera-XT DNA library prep kit (Illumina) following manufacturer's recommendations. Amplicons of the corresponding regions were prepared by PCR reaction with Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) using custom-designed primers for each exon (primer sequence available upon request). Deep sequencing was performed on the MiSeq System (Illumina) at 150 bp read length in paired-end mode, reaching an average depth of coverage of 9900X.

Bioinformatics Analysis

Amplicon sequencing was analyzed using a customized pipeline. For Illumina workflow, demultiplexing was performed with Miseq Reporter 2.6 (Illumina) and the paired-end reads were aligned on GRCh38 human reference genome. BAMClipper tool was adopted to perform soft-clipping in order to remove amplicon primers from alignment. Single nucleotide variants were called with SNVMix2 tool while insertions and deletions

were called with the HaplotypeCaller function of GATK3 adopting a combination of optional parameters suited to detect variation with low-allele-frequency small mapping quality (–minimum-mapping-quality 10; –max-alternate-alleles 1; –sample-ploidy 8; –max-reads-per-alignment-start 1,000). All variants were annotated with Annovar and filtered according to Exac minor allele frequencies, 0.1 altered allele fraction and at least a total depth of coverage of 20X and 5X of the altered allele. Moreover, variants detected in only one strand were considered as FFPE artifacts.

RNA- Sequencing

Total RNA was extracted using RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and used for cDNA library synthesis using TruSeq RNA Exome kit (Illumina) according to manufacturer's instructions. Single cDNA libraries were pooled and hybridized to a set of probes covering 45 Mb of coding exonic regions. Paired-end libraries were then sequenced at 2×80 bp on a NextSeq500 instrument (Illumina), producing an average of 51.1×10^6 reads per sample. After FASTQ generation and trimming of low-quality bases and sequencing adapters, paired-end reads were aligned with the TopHat/BowTie pipeline and gene expression was quantified with the package HTSeq-count and normalized as count per million (CPM) using the R-bioconductor pakage edgeR. The set of genes differentially expressed (p-value $< 10^{-3}$) between KIT-mutant and quadruple-WT GIST was obtained with the R-bioconductor package *limma* (lmfit an eBayes functions). The list of selected genes was used to perform hierarchical clustering of the low-allele-fraction KIT-mutant sample with the R-bioconductor package pheatmap (clustering distance: correlation; clustering method: complete).

PCR, qPCR, and Sanger Sequencing

KIT exon 9 and 11 were re-sequenced on FFPE tumor specimens using the Sanger sequencing method on ABI 3730 Genetic Analyzer (Applied Biosystems, Monza, Italy). Primer pairs, designed with Primer Express 3.0 Software (Applied Biosystems), were specific to amplify exons and part of the flanking intronic regions. PCR products were sequenced on both strands using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) on a ABI 3730 Genetic Analyzer (Applied Biosystems).

FGF4 copy number status was measured on ABI Prism 7900HT platform (Applied Biosystems) using FAM-labeled TaqMan Copy Number Assays (Thermo Fisher Scientific) targeting FGF4 (Hs02374436_cn) and XXRA1 (Hs03782780_cn), located in chromosome bands 11q13.3 and 11q13.4, respectively. TaqMan RNaseP Control Reagent (VIC-labeled) was used as internal reference control. Estimation of FGF4 copy number was done using DDCt method in comparison with XRRA1 and with a normal diploid sample as a calibrator.

Immunohistochemistry

Immunohistochemical analysis for CD117/c-Kit was performed on 3 μm paraffin-embedded tumor sections using monoclonal pre-diluted anti-CD117 clone YR145 (Ventana Medical Systems, USA) on Ventana Benchmark Ultra platform. Antigen Retrieval was performed in UltraCC1 Tris-HCl buffer pH 8.2–8.5 at 95°C for 24–48 min, and the immunologic reaction was visualized with the OptiView DAB Detection Kit (Ventana, USA).

RESULTS

The series consisted of 26 GIST specimens selected as negative for KIT/PDGFRA/BRAF/NRAS/KRAS mutations and with intact SDH complex, whose molecular characterization was performed by Sanger sequencing and immunohistochemistry. These samples were analyzed by means of a custom NGS amplicon approach targeting key genes frequently altered in GIST (KIT, PDGFRA, BRAF, NRAS, KRAS, SDHA, SDHB, SDHC, SDHD, and NF1), reaching an average depth of coverage of 295X. Overall, three samples carrying NF1 loss-of-function mutations were identified, and therefore excluded from further analyses (**Table 1**). These tumors were found to carry clearly pathogenic mutations, either truncations (p.Q519X and Q959X in GIST_406 and GIST_251 respectively) or frameshift mutations (p.R1241fs in GIST_203).

More interestingly, among the 23 remaining cases, five patients (22%) were unexpectedly found to carry pathogenic alterations in the KIT gene (**Table 1**). One case (GIST_169) showed a large deletion of 32 nucleotides (c. 1648_1672del) overlapping the intron-exon boundary upstream of exon 11 (**Table 1**). This deletion removes the 5′-splice site, and introduces a new donor splice site, coupled to the deletion of the first nine amino acids from the mature protein. Likely this event is not routinely detected by molecular diagnostic procedures since the deletion removes seven nucleotides from the flanking intronic sequence, where usually sequencing primers are located. The deletion was confirmed through Sanger sequencing using appropriate primers (**Supplementary Figure 1**).

The other four samples were instead carriers of a low-allele-fraction KIT mutation, with a detected altered allele frequency of 12–16% (**Table 1**). Three mutations affected KIT exon 11: a missense p.W557R mutation in GIST_260 and two non-frameshift alterations (p.L576_R588dup and p.Q575delinsQLPYE) in GIST_218 and GIST_307. The other mutation detected was p.S501delinsSAY involving exon 9 in GIST_241. These four events were clearly below the detection limit of conventional Sanger sequencing, even if the mutations were noticeable in the electropherogram at the level of background signal modifications (**Figures 1A–D**). The presence of these low-allele-fraction mutations was confirmed also through an independent NGS assay, based on deep sequencing of PCR amplicons targeting only KIT exon 9 and 11. This approach, that reached a minimum coverage of 9900X per sample, yielded very similar KIT-mutant allelic frequencies in GIST_241 and GIST_260, with a ratio of 9 and 23%, respectively (**Table 1**, *in brackets*). Besides, since this targeted sequencing approach uses different primers pairs to amplify KIT exons, we can rule out that the low ratio of the mutant allele is due to an artificial allelic dropout during DNA amplification.

To ensure that the tumor area was correctly isolated and dissected prior to nucleic acid extraction, we performed histopathological revision of the FFPE blocks of three of the four cases harboring low-allele-fraction KIT mutations

TABLE 1 | List of pathogenic mutations identified by targeted deep sequencing.

ID	Gene	Mutation (exon, cDNA, protein)	Type of mutation	Depth of coverage	Mutant allele frequency
GIST_406	NF1	Exon 22, c.C2875T, p.Q959X	Stop Gain	123X	47%
GIST_251	NF1	Exon 14, c.C1555T, p.Q519X	Stop Gain	213X	37%
GIST_203	NF1	Exon 28, c.3721delC, p.R1241fs	Frameshift deletion	403X	93%
GIST_260	KIT	Exon 11, c.T1657C, p.W557R	Missense	396X (18379X)	**14%** (23%)
GIST_241	KIT	Exon 9, c.1502_1503insTGCCTA, p.S501delinsSAY	In-frame insertion	391X (5763X)	**12%** (9%)
GIST_307	KIT	Exon 11, c.1723_1724insAACTTCCTTATG, p.Q575delinsQLPYE	In-frame insertion	468X	**16%**
GIST_218	KIT	Exon 11, c.1726insC;1726_1764dup, p.L576_R588dup	In-frame insertion	274X	**12%**
GIST_169	KIT	Exon 11, c. 1648_1672del, p.550_558del	In-frame deletion	100X	49%

The table lists the depth of coverage and the mutant allele frequency of the TSCA target sequencing assay. In brackets there are the same values for the KIT exon 9 and exon 11 Nextera-XT PCR amplicon assay.
Bold values indicate the samples with low-allele-fraction mutations.

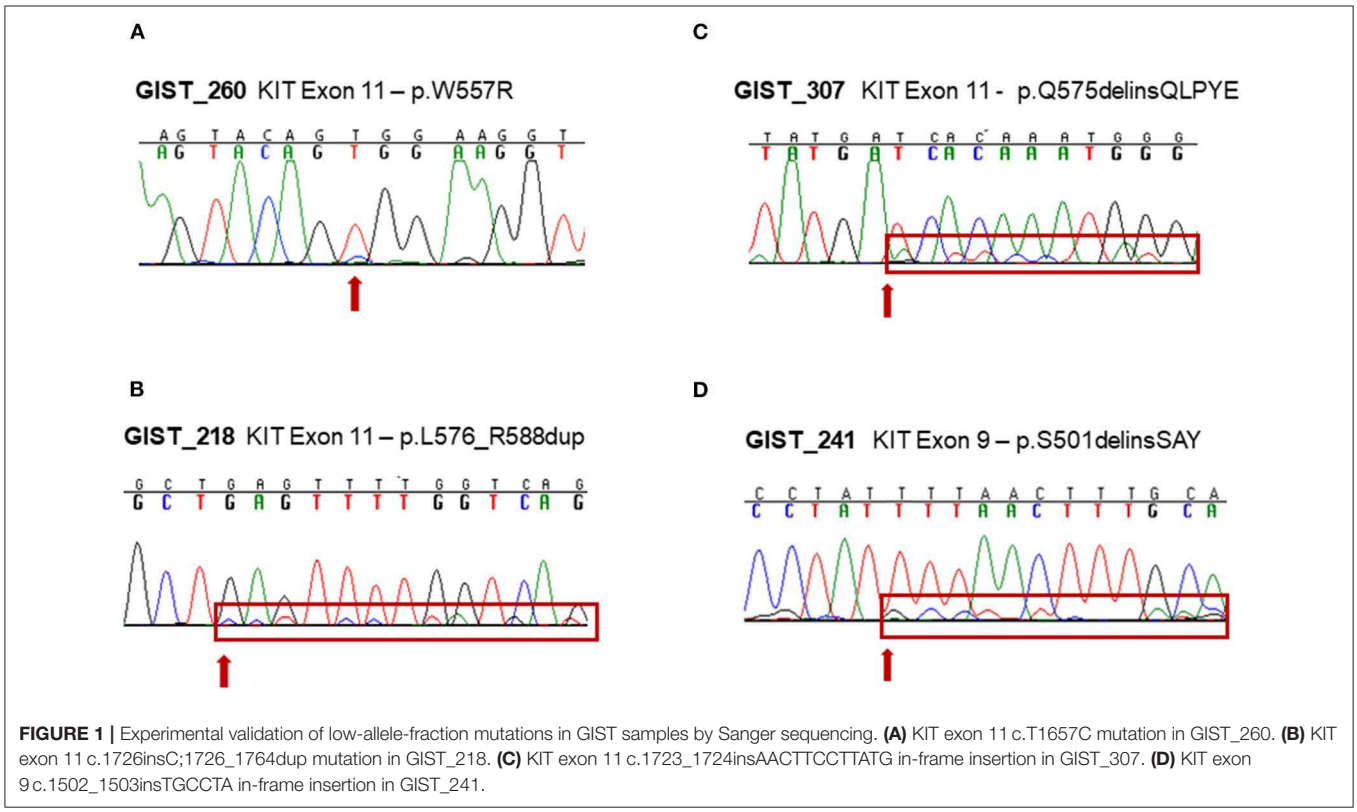

FIGURE 1 | Experimental validation of low-allele-fraction mutations in GIST samples by Sanger sequencing. **(A)** KIT exon 11 c.T1657C mutation in GIST_260. **(B)** KIT exon 11 c.1726insC;1726_1764dup mutation in GIST_218. **(C)** KIT exon 11 c.1723_1724insAACTTCCTTATG in-frame insertion in GIST_307. **(D)** KIT exon 9 c.1502_1503insTGCCTA in-frame insertion in GIST_241.

(GIST_260, GIST_241 and GIST_307). An expert pathologist selected again the tumor area containing more than 90% of tumor cells (**Figures 2A–C**) and DNA was extracted and sequenced by Sanger method. The presence of the low-allele-fraction mutation was confirmed in both cases, with profiles comparable to the ones resulting from the previous nucleic acid extraction, confirming that these alterations were indeed low frequency alleles (*data not shown*). CD117 immunostaining was strongly positive in all patients, as expected since almost all GISTs show CD117 expression. Interestingly, GIST_260 and GIST_307 additionally showed a combined membranous, cytoplasmic, and paranuclear Golgi-like positivity, suggestive of a diffuse alteration of KIT expression in the tumor mass (**Figures 2D–F**). It is noteworthy that Golgi-like staining, that is significantly more frequent in KIT-mutant than in WT-GIST (21), was detected in two low-allele-fraction mutant samples.

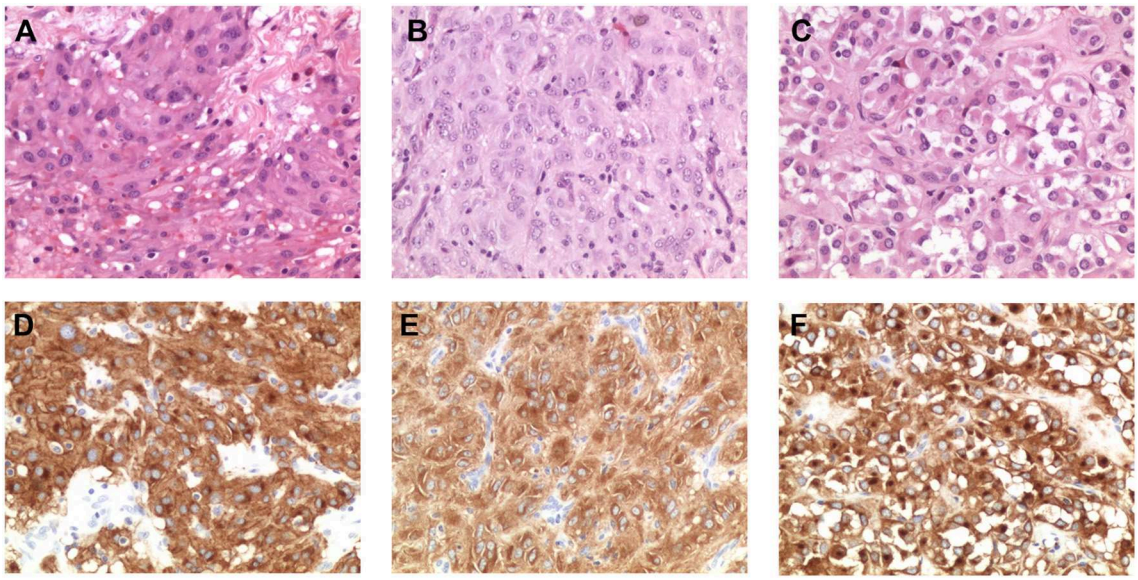

FIGURE 2 | Morphological and immunohistochemical analysis of low-allele-fraction KIT-mutant GIST. **(A)** GIST_260 appears as an epithelioid gastrointestinal stromal tumor comprising polymorphous cells arranged in nests and sheets with eosinophilic cytoplasm and peripherally placed nuclei, with mild nuclear atypia and no necrosis. The mitotic rate is 3/50 high power fields (HPF). (Hematoxylin and eosin stain: original magnification x 200). **(B)** GIST_241 is composed by spindle cell arranged in short fascicles and whorls, with pale eosinophilic fibrillary cytoplasm, ovoid nuclei, and ill-defined cell borders. The mitotic rate is 20/50 HPF. (Hematoxylin and eosin stain: original magnification x 200). **(C)** GIST_307 appears as an epithelioid gastrointestinal stromal tumor, composed by round cells with clear to eosinophilic cytoplasm arranged in sheets and nests, with low-grade nuclear atypia, mild nuclear pleomorphism and indistinct nucleoli. The mitotic rate is 2/50 HPF. (Hematoxylin and eosin stain: original magnification x 200). **(D)** CD117/KIT staining in GIST_260 reveals a strong positivity with membrane, cytoplasmic and "dot-like" Golgi staining (original magnification x 200). **(E)** KIT staining in GIST_241 shows a diffuse cytoplasmic staining (original magnification x 200). **(F)** Immunohistochemical analysis of CD117 expression in GIST_307 shows a strong and diffuse cytoplasmic and paranuclear Golgi-like staining (original magnification x 200).

In one of the four cases (GIST_260) whole transcriptome sequencing and targeted KIT mRNA sequencing was performed, revealing a high expression of the mutant allele, despite the low allelic fraction at the DNA level (**Figure 3A**). Furthermore, this sample clustered with KIT-mutant samples with respect to the genes differentially expressed between quadruple-WT and KIT-mutant GIST (**Figure 3B**). Of relevance, GIST_260 did not express FGF4, that is selectively upregulated in quadruple-WT cases and is not expressed in KIT-mutant GIST (16). FGF4 copy number status was also measured in the low-allele-fraction samples, confirming the absence of FGF4 gain (**Supplementary Figure 2**), that we showed as a feature of quadruple-WT GIST (16).

Lastly, the clinical course of the four patients carrying low-allelic-fraction KIT mutations was analyzed, showing that one of the four patients (GIST_307) developed peritoneal metastasis during the disease course (**Table 2**). The patient was treated with imatinib for 3 years and the survival from the time of metastatic relapse lasted for 40.5 months, an interval that is comparable to the median survival time of KIT/PDGFRA-mutant metastatic patients (56.6 months) and definitely higher than that of quadruple-WT GIST (25.2 months) (**Supplementary Figure 3**), thus reinforcing the relevance of low-allele-fraction KIT mutations in driving TKI-response in GIST.

Collectively these data indicate that roughly one out of five patients diagnosed with a KIT/PDGFRA/SDH/RAS-P wild-type GIST is a *bona-fide* carrier of pathogenic KIT mutation, thus expected to be eligible for and responsive to the various therapeutic lines of TK-inhibitors approved for KIT/PDGFRA-mutant GIST.

DISCUSSION

In this study we analyzed a series of 26 GIST negative for KIT/PDGFRA/BRAF/NRAS/KRAS mutations and with intact SDH complex, analyzed in two different Diagnostic Centers (local and referral), identifying three NF1-mutated samples, in agreement with a previous study showing a relevant frequency of NF1 mutations in quadruple-negative GIST cases (11). Quite unexpectedly, we identified five cases carrying pathogenic KIT mutations, which means that a fraction of more than one out of five apparently quadruple-WT GIST actually turns out to be KIT–mutant. Thus, our results demonstrate that a significant fraction of GIST patients actually affected by a KIT–mutant tumor are missed by the state-of-the-art molecular diagnostic protocols due to the limits of the standard techniques in use. Therefore, in practice, the fraction of patients affected by a KIT/PDGFRA WT GIST should be considered lower than currently expected. As a matter of fact, large deletions involving exon-flanking regions can be missed through allelic dropout while low-allele-fraction mutations are routinely overlooked by conventional Sanger sequencing due to the inherent detection limit of the

approach. Indeed, a low frequency KIT mutation was already reported in a previous study made with an amplicon sequencing approach, where an exon 11 V561D was described at 9% allele frequency in a GIST specimen (22). More importantly, a recent large scale genomic study of more than 5,000 tumor samples definitely showed that a low-allele-fraction mutations in cancer samples is a surprisingly frequent condition, with a routine detection of hotspot mutations in actionable genes such as EGFR, KRAS, PIK3CA and BRAF with an allele fraction below 10% in about 20% of clinical samples (23). Besides, in this study the authors also show that cases with low mutant allele frequency respond to TKI target therapy at the same level of cases with high allele frequency mutations, indirectly proving that low frequency mutations are biologically meaningful and clinically actionable (23). Likewise, we showed that GIST_307, carrying a low-allele-fraction KIT mutation, exhibited a long-term response to imatinib, with an overall survival of more than 3 years after metastatic spread of the disease.

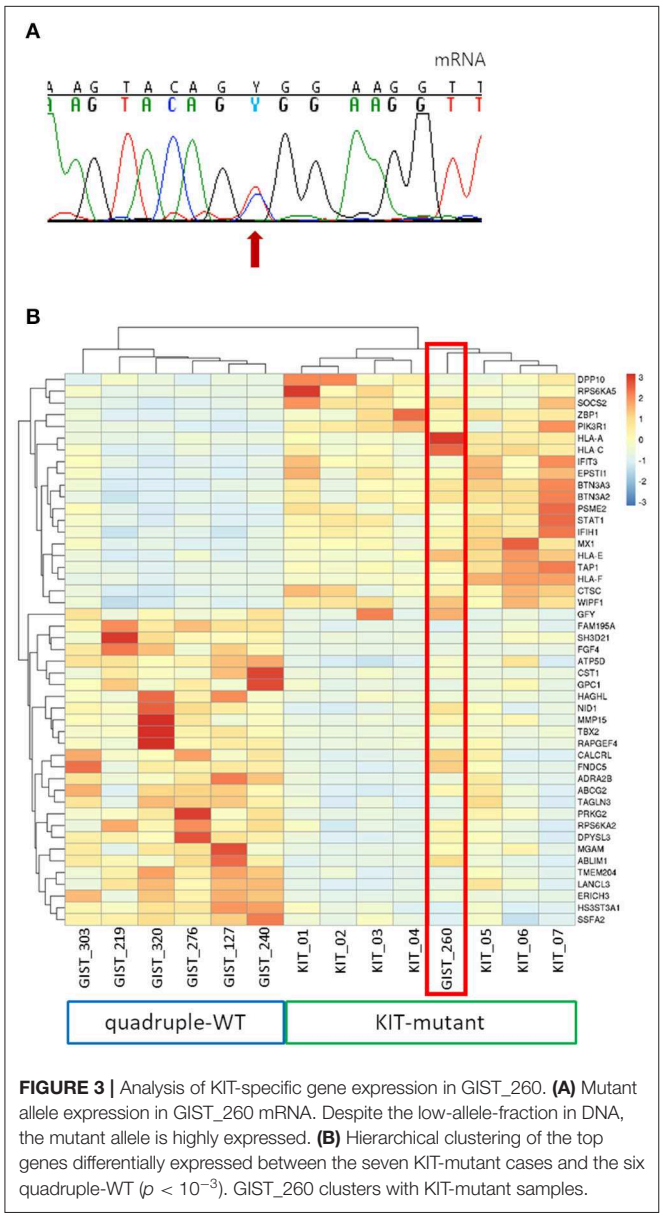

FIGURE 3 | Analysis of KIT-specific gene expression in GIST_260. **(A)** Mutant allele expression in GIST_260 mRNA. Despite the low-allele-fraction in DNA, the mutant allele is highly expressed. **(B)** Hierarchical clustering of the top genes differentially expressed between the seven KIT-mutant cases and the six quadruple-WT ($p < 10^{-3}$). GIST_260 clusters with KIT-mutant samples.

The reasons for the presence of low-allele-fraction mutations in cancer samples are various and complex, ranging from intra-tumor spatial heterogeneity to FFPE-induced degradation and chemical modification of DNA, that can impact on target amplification efficiency and reliability (24). Indeed, tumor heterogeneity is supposed to play a major role in low-allele-fraction mutations, an issue that can be highly relevant for necrotic tumors, since a recent study revealed an allele ratio of the same driver mutation in different samplings of the same GIST specimen from 10% to up to 60% (25). All these factors, coupled with the low sensitivity of Sanger sequencing, are supposed to play a role in the occurrence of low-allele-fraction mutations, that are supposed anyway to behave as clinically actionable alterations (23).

These findings underline once again the importance to refer patients with KIT/PDGFRA WT GIST to high-volume molecular diagnostic centers as already also suggested by the recent clinical guidelines (26), in which the implementation of appropriate next-generation-sequencing panels could be used to address the few cases with cryptic KIT mutations.

Quadruple-WT GIST represent an undefined and heterogeneous category of tumors (15, 27), that inevitably poorly respond to standard treatments, represented by TKI, due to the lack of the target oncogenic alteration. The detection of a significant fraction of this subgroup as carrier of actionable KIT mutations not only advocates the routine implementation of next generation sequencing approaches in the current molecular diagnostic protocols, but also opens new and effective therapeutic strategies for these patients, that are actually devoid of active pharmacological opportunities. As a matter of fact, our findings suggest that, in the metastatic setting, patients with a diagnosis of a KIT/PDGFRA WT GIST, except for those with

TABLE 2 | Clinical and demographic data of the low-allele-fraction KIT-mutant patients.

ID	Age range	Site	Size (cm)	Mitotic count	Risk classification	Metastasis	Status*
GIST_260	51–55	Jejunum	11	2	High	No	NED
GIST_241	71–75	Ileum	11	>5	High	No	NA
GIST_307	61–65	Ileum	8.5	<5	High	Peritoneum	DOD
GIST_218	56–60	Ileum	7	4	Intermediate	No	AWD

**Patients' status at last follow up: NED, no evidence of disease; DOD, died of disease; AWD, alive with disease; NA, not available.*

known therapeutic molecular targets (involving BRAF or NTRK or FGFR), should always be treated with imatinib because the event of a cryptic KIT mutation may occur. In these cases, the predictive role of baseline and 1-month FDG-PET could assist the physicians in the early evaluation of imatinib response in clinical practice (28).

In conclusion, this analysis demonstrates that a significant proportion of quadruple–WT GIST patients are actually carrying pathologically relevant low-allele-fraction KIT mutations, that would benefit from TKI treatments both in the adjuvant and metastatic setting and that should be readily identified at the early diagnostic stage though implementation of appropriate next-generation-sequencing panels and addressing to national hub diagnostic centers. These results warrant further investigations to confirm in a wider series that in 20% of KIT/PDGFRA/SDH/RAS-pathway wild-type GIST it is possible to find cryptic KIT alterations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) (PRJNA602810).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical committee of Azienda Ospedaliero-Universitaria Policlinico S. Orsola-Malpighi. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MP, MU, and AAs: study concept and design. AAs, MU, AS, MN, MS, AD, AAl, and PS: acquisition of data. BV, DC, GG, and MP: patients' clinical supervision. VI: data analysis. AAs, MU, and MP: drafting of the manuscript. AAs, MU, VI, MN, MS, and MP: study supervision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00504/full#supplementary-material

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Synergy of GSK-J4 With Doxorubicin in KRAS-Mutant Anaplastic Thyroid Cancer

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Background: Anaplastic thyroid cancer is the most aggressive thyroid cancer and has a poor prognosis. At present, there is no effective treatment for it.

Methods: Here, we used different concentrations of GSK-J4 or a combination of GSK-J4 and doxorubicin to treat human Cal-62, 8505C, and 8305C anaplastic thyroid cancer (ATC) cell lines. The *in vitro* experiments were performed using cell viability assays, cell cycle assays, annexin-V/PI binding assays, Transwell migration assays, and wound-healing assays. Tumor xenograft models were used to observe effects *in vivo*.

Results: The half maximal inhibitory concentration (IC50) of GSK-J4 in Cal-62 cells was 1.502 μM, and as the dose of GSK-J4 increased, more ATC cells were blocked in the G2-M and S stage. The combination of GSK-J4 and doxorubicin significantly increased the inhibitory effect on proliferation, especially in KRAS-mutant ATC cells *in vivo* (inhibition rate 38.0%) and *in vitro* (suppresses rate Fa value 0.624, CI value 0.673). The invasion and migration abilities of the KRAS-mutant cell line were inhibited at a low concentration ($p < 0.05$).

Conclusions: The combination of GSK-J4 with doxorubicin in KRAS-mutant ATC achieved tumor-suppressive effects at a low dose. The synergy of the combination of GSK-J4 and doxorubicin may make it an effective chemotherapy regimen for KRAS-mutant ATC.

Keywords: anaplastic thyroid cancer, epigenetics, GSK-J4, synergistic action, KRAS-mutant

INTRODUCTION

The prognosis of anaplastic thyroid cancer (ATC) patients is poor, which have a median survival of 3–12 months (Subbiah et al., 2018; Lin et al., 2019). And their overall survival (OS) and survival rate have not been significantly improved in the past 40 years, suggesting that there is no effective treatment for improving long-term prognosis. Because of the aggressive nature and limited treatment methods, it is necessary to explore effective chemotherapies with less toxic side effects

(Ezaki et al., 1992; Arora et al., 2014; Hanley et al., 2015). Combination of doxorubicin and radiation for ATC treatment was widely accepted from 1980s (Kim and Leeper, 1983; Tennvall et al., 1994; Sun et al., 2013). Despite its cardiac toxicity, doxorubicin has been the most commonly used drug in ATC treatment (Sun et al., 2013; Fan et al., 2020). Doxorubicin was considered as the most effective drug for ATC until the randomized study of the Eastern Cooperative Oncology Group (ECOG) shew the combination of cisplatin and doxorubicin was more effective than doxorubicin alone (Denaro et al., 2013). At present, the key for ATC treatment is multimodal therapy. Several studies have shown that the combination of surgery, radiation therapy with chemotherapy (such as doxorubicin), might improve the 1-year OS to more than 40%(Baek et al., 2017; Prasongsook et al., 2017; Fan et al., 2020). Targeted therapy is another possible option, especially for patients with BRAF V600E mutation. Dapafenib combined with trametinib has been proved to have clinical activity (Kapiteijn et al., 2012; Hsu et al., 2014; Jin et al., 2017; Molinaro et al., 2017; Iyer et al., 2018; Subbiah et al., 2018). Whether targeted therapy is beneficial to the long-term survival of patients with ATC has not been determined (Raue and Frank-Raue, 2016; Cao et al., 2019; Ljubas et al., 2019).

In recent years, research on histone modification affecting tumorigenesis and development has provided a target for drugs. Histone gene modification can effectively regulate gene expression levels. However, not all modification types have a stable distribution. Lysine methylation was found to be enriched in the coding region, and each methylation site corresponded to a special distribution pattern (Jin et al., 2017; Molinaro et al., 2017). These relationships provide the possibility to study the relationship between histone methylation and oncogene expression. In the present study, we knew that H3K27me3 expression was upregulated in thyroid cancer, particularly in those with a less differentiated phenotype (Tsai et al., 2019).

The methyltransferase JMJD3, which generates H3K27me3 (trimethylated lysine 27 on histone 3) alterations, consists of a JmjC catalytic domain and a C terminus, which combine to form a larger binding area. JMJD3 was found to be associated with cell proliferation and differentiation, and its expression was elevated under the stimulation of inflammation, viruses, tumors, and other factors (Lin et al., 2008; Zhu et al., 2014). Elevating its expression level in specific types of tumors may trigger an immune response and thus promote the progression of tumors. Specific inhibitors of histone methyltransferases (HMTs) with lysine and arginine residues have been developed. GSK-J4, an inhibitor of JMJD3 (Jumonji domain-containing protein 3), is one of them. GSK-J4 is an ethyl ester derivative of the H3K27 methyltransferase inhibitor GSK-J1 (Di Desidero et al., 2017). GSK-J4 can regulate the expression of downstream genes, such as NOTCH1, TNF-a, and PTEN, by inhibiting the activity of JMJD3 and affecting cell proliferation and the expression of stem cell-related genes in cancer cells (Lin et al., 2012; Arora et al., 2014; Abdulghani et al., 2016; Broecker-Preuss et al., 2016; Bible and Ryder, 2016; Park et al., 2017; Lee et al., 2018).

In this manuscript, we used GSK-J4 and doxorubicin to treat human ATC cell lines (Cal-62, 8505C, and 8305C) and found that GSK-J4 significantly inhibited the proliferation of ATC cells. The combination of GSK-J4 and doxorubicin had a stable synergistic effect on KRAS-mutant cell lines, which inhibited sphere formation, tumorigenicity, migration, and invasion of cells at a low dose of doxorubicin. GSK-J4 combined with doxorubicin may be an effective chemotherapy regimen for ATC.

MATERIALS AND METHODS

Cell Lines

The human Cal-62, 8505C, and 8305C anaplastic thyroid cell lines used in this study were purchased from GuangZhou Jennio Biotech Co. Cal-62 is KRAS G12R mutated and BRAF wide type (WT), while 8305C and 8505C are BRAF V600E mutated. Cal-62 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 0% fetal bovine serum (FBS), penicillin (50 units/ml; Gibco), and streptomycin (50 μg/ml; Gibco) as previously described. 8505C and 8305C cells were cultured in Minimum Essential Medium (MEM) with 10% FBS, penicillin (50 unit/ml; Gibco), and streptomycin (50 μg/ml; Gibco). All cell lines were grown at 37°C in a 5% CO_2 atmosphere.

CellTiter-Glo® Luminescent Cell Viability Assay

Cell proliferation was measured by cell viability assays. For the cell viability assay, cells were seeded in a 96-well plate at a density of 3,000 cells/well. After recovering for 24 h, cells were treated with complete media alone or media containing GSK-J4 (maximum concentration = 20 μM, double dilution, TargetMol) or doxorubicin (maximum concentration = 10 μM, double dilution, Whiga) at different concentrations for 48 h. For the time-related cell viability assays, cells were recovered for 24 h and were then treated with complete media alone or GSK-J4 (1 μM), and cell viability was recorded every day. Cell viability was measured by using a CellTiter-Glo (CTG) mixture according to the manufacturer's instructions. The amount of ATP was directly proportional to the number of cells present in culture. ATP was quantified by using a fluorescence microplate. Viability was calculated using a background-corrected absorbance according to the following formula: viability (%) = A of experiment well/A of control well × 100. Dose reduction index (DRI) means a multiple reduction in the dose of a drug used at a level of inhibition produced by a combination of drugs over that used alone to achieve the same level of inhibition, and the calculation formula is DRI= $(Dx)_1/(D)_1$ or $(Dx)_2/(D)_2$.

To obtain the IC50 (half maximal inhibitory concentration), GraphPad Prism 6 software was used to analyze the data. Calcusyn 2.0 software was used to calculate the combination index (CI), DRI, and Fa value.

Transwell Migration and Invasion Assays

Transwell chambers (Guangzhou Sagene Technology Co.) with transparent PET membranes (8.0 μm pore size) were inserted

into a 24-well culture plate (Corning, NY 14831, USA). For the cell invasion assay, the upper surface of the PET membrane was equally covered with 100 μl of 1.25 mg/ml Matrigel (Shanghai Pharmaceuticals Holding Co.). Briefly, 300 μl of serum-free cell suspension containing 2×10^5 cancer cells was added to the upper chamber, and the cancer cells were treated with different concentrations of GSK-J4 (0, 1.25, 2.5, or 5 μM) or a combination of GSK-J4 or/and doxorubicin (DOX: GSK-J4 = 0.156:0.078 μM) at different concentrations. After incubation at 37°C in 5% CO_2 for 24 h, the cells were fixed with methanol and stained with crystal violet staining solution. On the upper surface of the membrane, remaining nonmigrating cells were cleared with cotton swabs. The migratory and invasive cells on the lower surface of the membrane in each chamber were counted randomly under high-power fields at least five times.

The migration rate of ATC cells was determined by a wound-healing assay. The cells were grown to confluence on six-well plates. A scratch was made through the cell monolayer using a 1,000 μl pipette tip. After washing with phosphate-buffered saline (PBS) three times, maintenance medium containing 0.2% FBS was added, and the cancer cells were treated with medium, GSK-J4 or doxorubicin alone, or a combination of GSK-J4 and doxorubicin (DOX: GSK-J4 = 0.156:0.078 μM). After making the scratch, images of the wounded area were captured immediately (0-h time point). The migration of cells into the wounded area was recorded once every 4 h for 24 h using an inverted microscope (Nikon).

Cell Cycle Analysis

After treatment with different concentrations of GSK-J4 for 48 h, the cells were harvested and resuspended in 500 μl of DNA staining solution and stained by the addition of 5 μl of permeabilization solution. After incubation at room temperature for 15 min, stained cells were immediately analyzed by flow cytometry.

Cellular Apoptosis Assays

Both adherent cells harvested by trypsinization and floating cells collected by centrifugation were used for the annexin-V/PI binding assay. After treatment with different concentrations of GSK-J4 for 48 h, the cells were harvested, resuspended in 500 μl of 1× binding buffer, and stained by adding 2.5 μl of FITC-annexin V and 5 μl of PI working solution. When detecting synergy, after treatment with GSK-J4 or/and doxorubicin (DOX: GSK-J4 = 0.156:0.078 μM), Cal-62 cells were collected and resuspended in 500 μl of binding buffer. After 5 μl of YO-PRO-1 and 5 μl of 7-AAD were added for each well. After incubation at room temperature in the dark for 10 min, stained cells were immediately analyzed by flow cytometry.

After treatment with GSK-J4 or/and doxorubicin (DOX: GSK-J4 = 0.156:0.078 μM), RIPA buffer with protease inhibitor cocktail (Roche 4693132001) was used for lysing Cal-62 cells. Samples were diluted with 0.25 volume to 5× SDS-PAGE Sample Buffer (GenStar). For making albumen denaturation, the proteins were heated for 8 min at 100°C. SDS-PAGE was used to perform Gel electrophoresis, and then proteins were transferred to Immun-Blot PVDF Membrane (Bio-Rad). The primary antibodies used were caspase 3 (1:1000, Affinity Cat# AF6311), Pro-caspase 3 (1:1000, Abcam Cat# ab32150), and β-actin (1:3000, Proteintech Cat# 60008). Membranes were incubated with aforementioned primary antibodies for 16–20 h at 4°C, and then incubated for 1 h with peroxidase conjugated secondary antibodies (1:10,000, Abcam). Finally, chemiluminescence detection was performed and at least repeated for three times.

Tumorsphere Culture

After trypsinization to obtain a single-cell suspension, serum free medium was used to resuspend human Cal-62 thyroid cancer cells. Cells were seeded (5,000 cells/5,000 μl/well) in 24-well low-attachment plates with Serum free medium in triplicate. After treatment with medium, GSK-J4 or doxorubicin alone or a combination of GSK-J4 and doxorubicin (DOX: GSK-J4 = 0.156:0.078 μM), the number of spheres in each well was counted after 6 d of incubation.

Tumor Xenograft Models

Female BALB/c nude mice (6–8 weeks old, weight > 18 g) were obtained from the animal core facility of Nanjing Medical University. Mice were raised under specific pathogen-free conditions according to protocols approved by the animal laboratory of Zhongshan School of Medicine. After 1 week of adaptation, mice were injected subcutaneously in the axillary region with 1.4×10^6 Cal-62 cells in 200 μl of serum-free media. The mice implanted with tumor cells were randomly distributed into four groups (n = 3 per group) and received GSK-J4 alone, doxorubicin alone, a combination of GSK-J4 and doxorubicin, or vehicle (PBS) by intraperitoneal injection once daily at a dose of 0.25 ml/10 g body weight. The mice were treated with the above strategy continuously for 14 d, and tumor volume was recorded every 2 d by caliper measurement of tumor diameter and calculated according to the following formula: $V = L \times W^2/2$ (L, length; W, width). Fourteen days after treatment, the mice were sacrificed, and the tumors were resected and weighed. All animal experiments were conducted according to the "Guidelines for the Welfare of Animals in Experimental Neoplasia". This study was carried out in accordance with the principles of the Basel Declaration and the recommendations of the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for avoiding or reducing animal experiments and the suffering of laboratory animals. The animal research protocol was approved by the ethical committee of the First Affiliated Hospital of Sun Yat-sen University.

Statistical Analyses

All the *in vitro* experiments were repeated at least three times. Continuous variables were represented as mean ± standard deviation (SD). The significance of differences between samples *in vitro* assays was determined by Student's t-test. In animal experiments, two-way repeated measures analysis of variance (ANOVA) was used to compare the differences among groups. In all the statistical analyses, $p < 0.05$ is considered to be statistically significant.

RESULTS

GSK-J4 Inhibits the Proliferation of Human ATC Cells

The antiproliferative effect of GSK-J4 and doxorubicin on ATC cells was measured by a cell viability assay. The data indicated that GSK-J4 efficiently inhibited the proliferation of ATC cells. After treatment for 48 h, the half maximal inhibitory concentrations (IC50s) of GSK-J4 in Cal-62, 8505C, and 8305C cells were 1.502, 5.269, and 5.246 μM, respectively (**Figure 1A**), and the IC50s of doxorubicin in Cal-62, 8505C, and 8305C cells were 0.100, 1.309, and 1.314 μM, respectively (**Figure 1B**). GSK-J4 had a continuing impact on Cal-62 cells over time (**Figure 1C**, $p < 0.05$). The results of the cell cycle analysis indicated that more ATC cells were blocked in G2-M and S phase with increasing drug concentrations (**Figure 1D**). These results suggest that GSK-J4 may cause cell damage, resulting in DNA replication being blocked. And the results of the apoptotic test showed that treatment with GSK-J4 induces cell apoptosis (**Figure 1E**, $p < 0.05$).

The Combination of GSK-J4 and Doxorubicin Inhibits the Proliferation of Cal-62 Cells

We treated ATC cell lines with the combination of GSK-J4 and doxorubicin, which showed a similar effect in 8505C and 8305C cells. With increases in the concentration of GSK-J4 and doxorubicin, the antagonistic effect first changed to a synergistic effect, but with continued increases in the concentration, the effect changed back to an antagonistic one. Thus, the two drugs exhibited synergy over a narrow concentration interval. The synergistic effect was slightly stronger in 8305C cells than in 8505C cells. When the two drugs acted on Cal-62 cells, the synergistic effect was obvious at lower concentrations than those seen in other cell lines, and the effect was stronger than that seen in the other two cell lines (**Figure 2A**). Computerized simulation of DRI indicated that at 75–97% growth inhibition levels, the doses of GSK-J4 could be reduced by 138.51-fold and 367.02-fold and the doses of doxorubicin by 1.63-fold and 2.67-fold in Cal-62 cells, the doses of GSK-J4 could be reduced by 16.66-fold and 38.90-fold and the doses of doxorubicin by 1.19-fold and 1.85-fold in 8505C cells, the doses of GSK-J4 could be reduced by 11.97-fold and 17.93-fold and the doses of doxorubicin by 1.09-fold and 2.58-fold in 8305C cells, respectively, when the drugs are used in combination (**Figure 2B**). We selected the ratio (DOX: GSK-J4 = 2:1) and drug concentration ratio (DOX: GSK-J4 = 0.156:0.078, Fa value 0.624, CI value 0.673) with the strongest synergistic effects to use for subsequent experiments (**Supplemental Tables 1–3**). Tumorsphere culture was performed in a KRAS-mutant cell line (Cal-62) to evaluate the suppressive abilities of the combination of GSK-J4 and doxorubicin. The combination of GSK-J4 and doxorubicin inhibited the 3D sphere growth of Cal-62 cells (**Figure 2C**). The number of tumorspheres per well

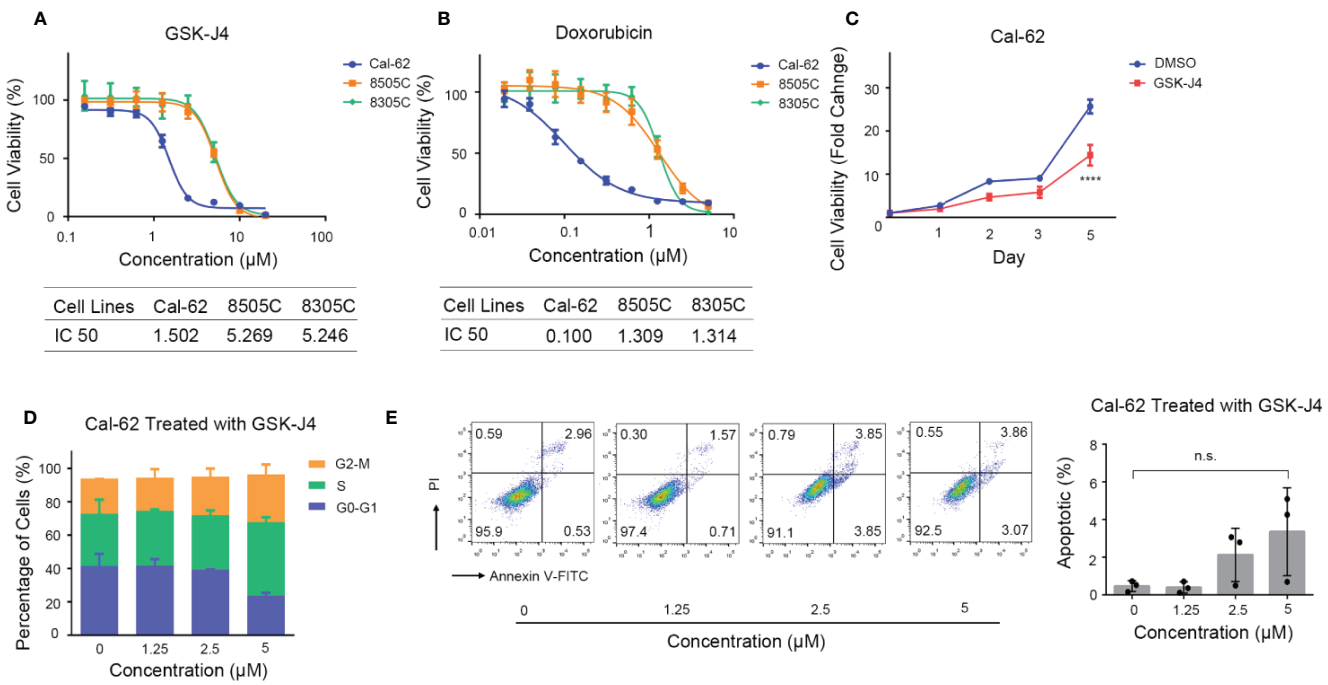

FIGURE 1 | Effect of GSK-J4 on the Cal-62 Anaplastic Thyroid Carcinoma Cell Line. The proliferation relationship between concentration of GSK-J4 **(A)** and doxorubicin **(B)** in anaplastic thyroid cancer cell lines. **(C)** Cal-62 cell proliferation in different treatment time of GSK-J4. Effects of GSK-J4 on the cell cycle **(D)** and apoptosis **(E)** in Cal-62 cells. IC50, half maximal inhibitory concentration.

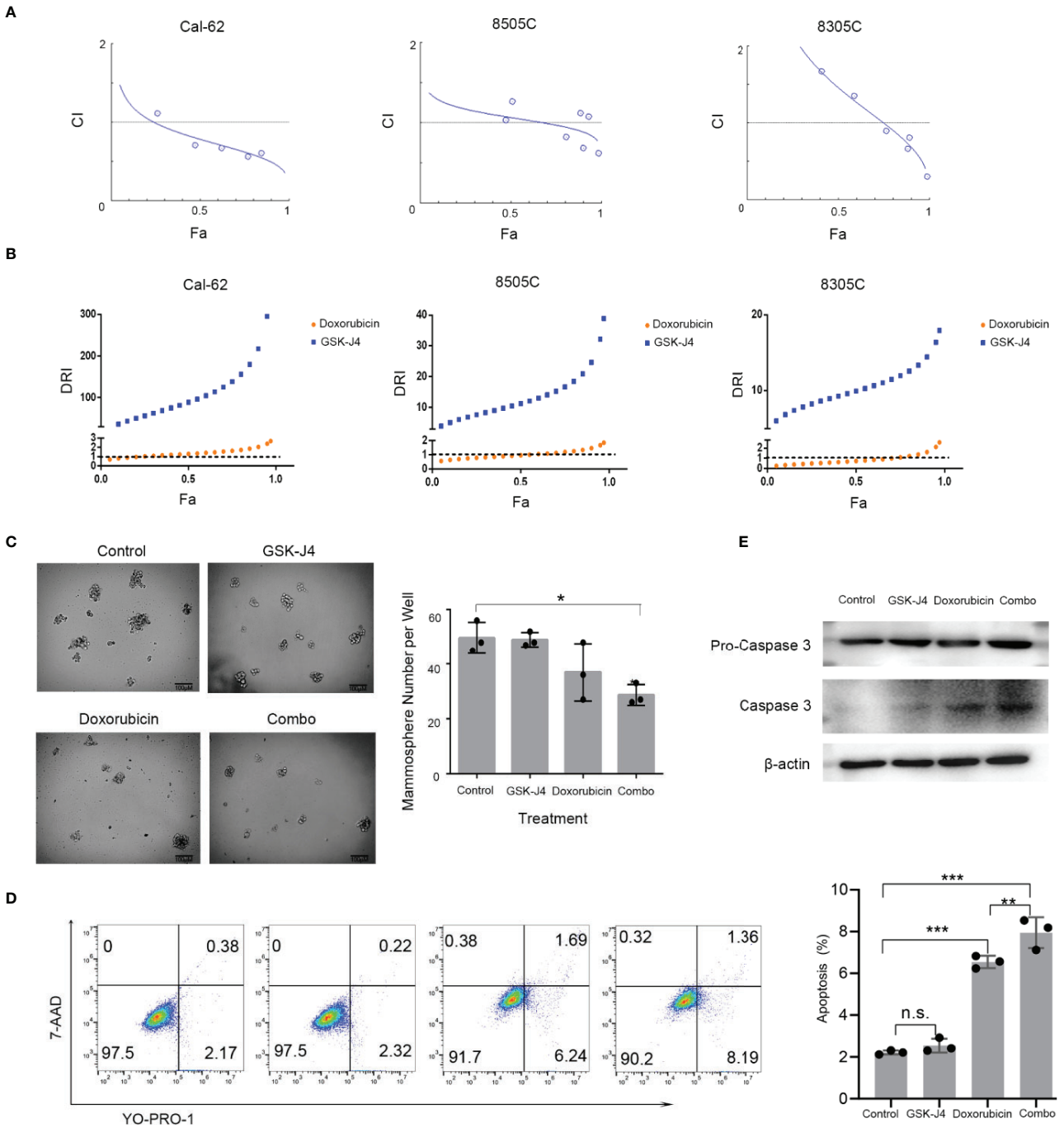

FIGURE 2 | The Effect of GSK-J4 Combined With Doxorubicin. Synergistic curves of Cal-62, 8505C, and 8305C cell lines treated with GSK-J4 and doxorubicin **(A)** DRI of Cal-62, 8505C, and 8305C cell lines treated with GSK-J4 and doxorubicin **(B)** The effect of GSK-J4 combined with doxorubicin on the sphere-forming ability **(C)** of the Cal-62 cell line. Scale bar, 100 μM. YO-PRO-1/7-AAD staining show the effect of GSK-J4 combined with doxorubicin on cell apoptosis of the Cal-62 cell line **(D)**, Western blot showed the caspase 3 and Pro-caspase 3 with the treatment of effect of GSK-J4 combined with doxorubicin **(E)** (DOX: GSK-J4 = 0.156:0.078 μM). DRI, Dose reduction index. n.s., no statistical difference. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

treated with GSK-J4 alone, doxorubicin alone, and the combination was 49 ± 3, 37 ± 11, and 29 ± 4, respectively, while that of the control group was 50 ± 6 (**Figure 2C**). In addition, from the cell images, we can see that the average diameter of the tumorspheres treated with the combination was significantly smaller than that of the tumorspheres in the control group (**Figure 2C**). To explore the combination effect on apoptosis, we tested YO-PRO-1/7-AAD staining cell in different

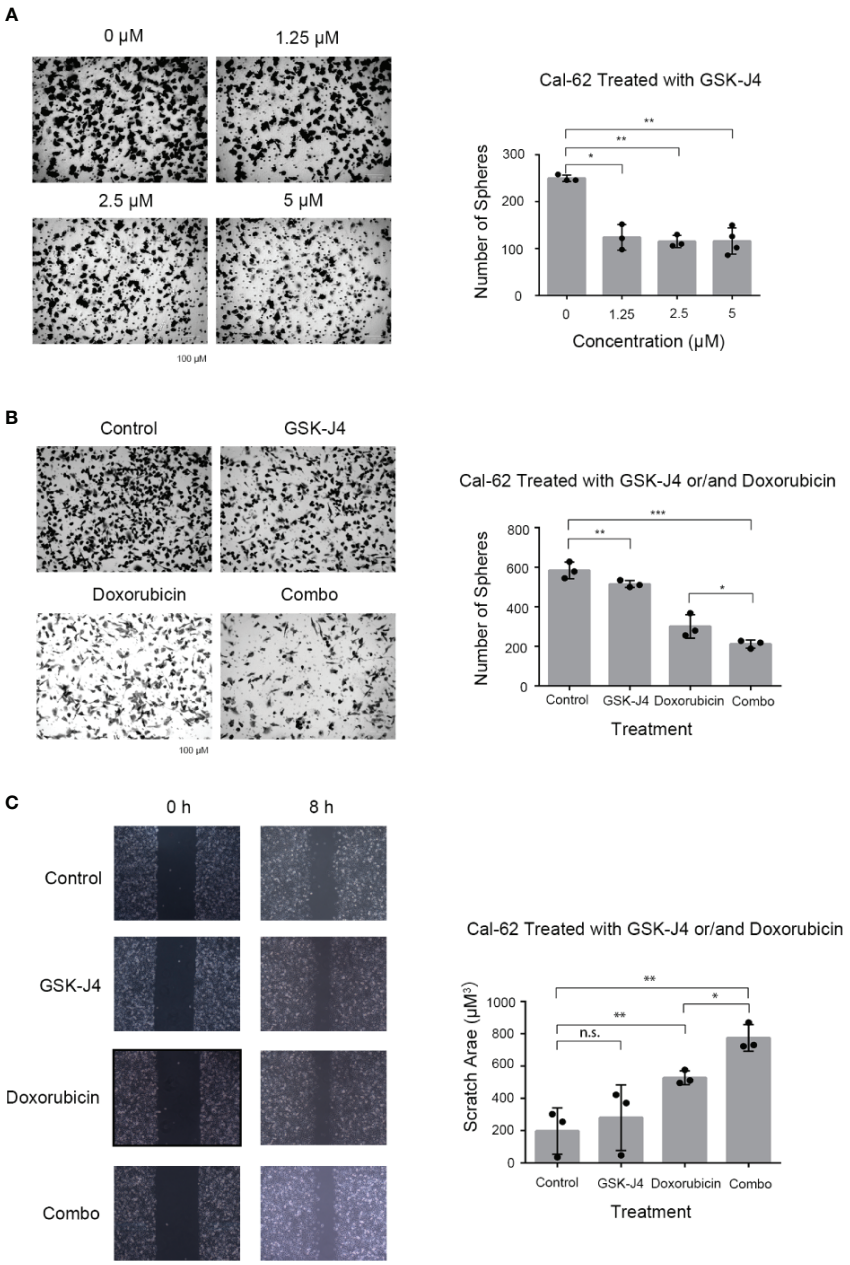

FIGURE 3 | Effects of GSK-J4 and Doxorubicin on Invasion and Migration of the Cal-62 Cell Line. The invasion ability of GSK-J4 in different concentration on Cal-62 cell line **(A)** the effect of GSK-J4 combined with doxorubicin on the invasion ability **(B)** and migration ability **(C)** of the Cal-62 cell line. Scale bar, 100 μM. n.s., no statistical difference. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

treatment groups and found apoptosis rate in the combined group increased significantly (**Figure 2D**). And combination of GSK-J4 and doxorubicin synergistically increased the caspase 3 level (**Figure 2E**). These data suggest that the combination of GSK-J4 and doxorubicin suppresses the sphere-forming abilities and growth of human ATC cells through inducing cellular apoptosis.

The Combination of GSK-J4 and Doxorubicin Inhibits the Migration and Invasion of Cal-62 Cells

Transwell chamber assay results showed that the number of migratory cells was significantly ($p < 0.05$) reduced in Cal-62 cells that were treated with GSK-J4 when compared with the number in nontreated cells (**Figure 3A**). The number of cells that

migrated per well in groups treated with 1.25, 2.5, or 5 μM GSK-J4 was 163 ± 10, 155 ± 9, and 158 ± 8, respectively, while that of the control group was 207 ± 11 (**Figure 3A**, $p < 0.05$). These data suggest that GSK-J4 inhibits migration in human thyroid cancer cells in a dose-dependent manner. In addition, when Cal-62 cells were treated with a single drug or a combination of both, the number of cells that migrated per well treated with GSK-J4, doxorubicin, or both was 515 ± 10, 312 ± 28, and 212 ± 12, respectively, while that of the control group was 584 ± 24 (**Figure 3B**, $p < 0.05$).

Scratch/wound-healing assays were performed in Cal-62 cell lines to evaluate the inhibitory effect of the combination of GSK-J4 and doxorubicin on tumor cell migration (**Figure 3C**). The data indicated that cell monolayer healing after 8 h was delayed in Cal-62 cells treated with a combination of GSK-J4 and doxorubicin when compared with nontreated cells and cells treated with a single drug alone (**Figure 3C**, $p < 0.05$).

Treatment With a Combination of GSK-J4 and Doxorubicin Inhibits the Growth of Cal-62 Cell Xenografts in Nude Mice

We investigated the antitumor effect of treatment with a combination of GSK-J4 and doxorubicin in nude mice bearing Cal-62 ATC xenografts. Intraperitoneal injection of a combination of GSK-J4 and doxorubicin every 2 d produced a significant sustained inhibitory effect (**Figure 4A**). The data showed that the growth of tumors in the groups treated with the combination of GSK-J4 and doxorubicin was significantly slower than that in the control group, GSK-J4 alone group, or doxorubicin alone group (**Figures 4B, C**). The inhibition rate was 38.0% in the groups treated with a combination of GSK-J4 and doxorubicin ($p < 0.05$). There were no obvious effects on the body weight of mice in the animal studies described above (data not shown), indicating that the combination of GSK-J4 and doxorubicin is likely well tolerated.

DISCUSSION

In this study, GSK-J4 significantly inhibited the proliferation of ATC cells, and the combination of GSK-J4 and doxorubicin had a stable synergistic effect in KRAS-mutant cell lines, which allowed for the inhibition of the sphere-forming abilities, tumorigenicity, migration, and invasion of the Cal-62 cell line at a low dose of doxorubicin.

Cal-62 cells have KRAS mutation and wild-type BRAF (KRAS G12R and BRAF wt, respectively). These alterations are common among anaplastic types of the disease, but few studies have been carried out. Additionally, 8505C and 8305C cells have BRAF gene mutations (Hoffmann et al., 2006; Sasanakietkul et al., 2018). Many new studies have been carried out on ATC patients with BRAF mutations, such as targeted therapy (Iyer et al., 2018; Knauf et al., 2018; Subbiah et al., 2018; ElMokh et al., 2019) and immunotherapy (Cabanillas et al., 2018). Although dabrafenib combined with trametinib has been widely accepted as an effective method for the treatment of BRAF mutation, there is no consensus that this therapy can be used for the treatment of KRAS mutant patients.

At present, the chemotherapeutic drugs are still one of the most important treatment for ATC, such as anthracyclines (doxorubicin) and paclitaxel (taxol and docetaxel) (Kouzarides,

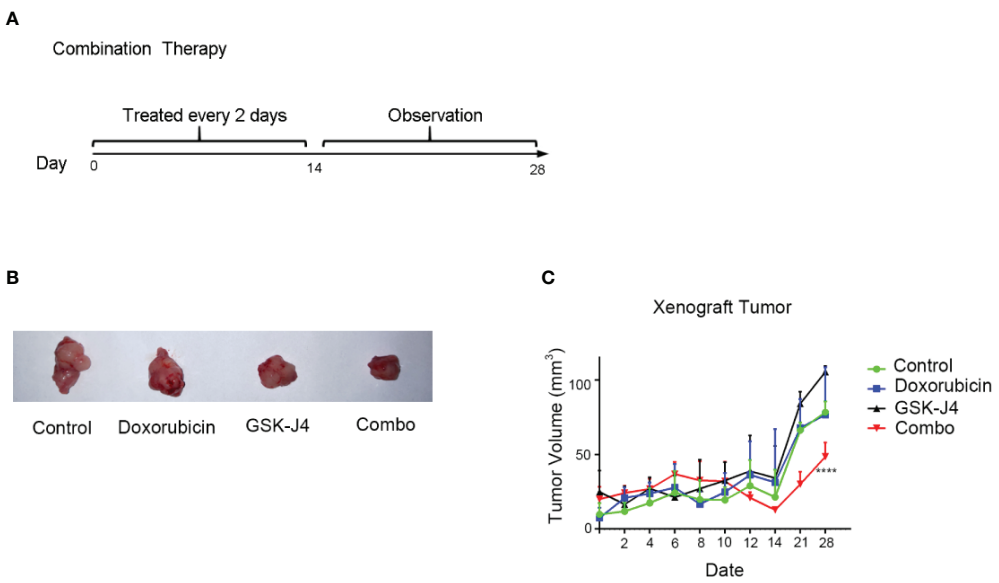

FIGURE 4 | Effect of GSK-J4 combined with doxorubicin on the tumorigenic ability of Cal-62 cells. The schematic diagram of *in vivo* experiment **(A)** tumor tissues **(B)** and volume **(C)** treated by different experimental groups.

2007; Kubicek et al., 2007; Helin and Dhanak, 2013). Moreover, it has been confirmed that the JAK-STAT signaling pathway is activated in RAS-positive ATC (Yoo et al., 2019). Aziz Zaanan et al. found that KRAS mutation increased the level of BCL-XL expression by elevating the level of STAT3 (signal transducer and activator of transcription 3) (Lin et al., 2013). Increased expression of BCL-XL was associated with decreased sensitivity of cells to chemotherapeutic drugs.

Because single drug cannot receive satisfactory results. The synergistic treatment of the two drugs is another option to improve the clinical efficacy. Several studies have reported that a combination of two or more drugs may benefit patients with anaplastic disease (Saini et al., 2018; Cao et al., 2019; Chintakuntlawar et al., 2019; Schurch et al., 2019). For example, combination sorafenib with quinacrine (Ha et al., 2017) and a combination of the BH3 mimic drug ABT-737 and doxorubicin (Ntziachristos et al., 2014) induced ATC cell apoptosis. Yong Sang Lee et al. tested primary cells cultured from ATC patients and found that different combinations of HNHA (a histone deacetylase), lenvatinib (a fibroblast growth factor receptor inhibitor), and sorafenib (a tyrosine kinase inhibitor) were more effective than single drugs (Hjelmeland et al., 2017). However, these discoveries are still in the basic experimental stages, and there are still many problems to be solved before their actual clinical application. Research related to clinical treatment has mainly focused on the efficacy of single-drug chemotherapy (Hu et al., 2012; Del Rizzo and Trievel, 2014; Ljubas et al., 2019), but these studies have not discussed the reasons for the poor efficacy of single drugs in treatment. The most effective drug, doxorubicin, has side effects, such that the dosage is strictly limited. In addition, some cancer cells easily resist doxorubicin. Even with doxorubicin chemotherapy, most patients still cannot avoid disease progression in the course of treatment. In cases in which existing single-drug therapies cannot effectively inhibit cancer progression, choosing two drugs with a synergistic effect to achieve a better therapeutic outcome than what would be achieved with a single drug while reducing the toxic side effects of the drugs may be another therapeutic option.

In the previous literature review, we knew that KRAS mutation upregulates the expression of STAT3. Maureen M. Sherry-Lynes et al. found that the transcription regulator STAT3 can directly bind to the JMJD3 promoter, specifically inducing JMJD3 expression and resulting in demethylation of H3K27me3, thereby affecting the expression of downstream genes that have been proven to be related to chemosensitivity. Ken Shiraiwa et al. suggested that STAT3 played an important role in ATC stem cells (Shiraiwa et al., 2019). After treatment with a STAT3 inhibitor, cancer cells were sensitized to chemotherapeutic drugs (Borbone et al., 2010). Thus, GSK-J4 is likely to block the effect of STAT3 on JMJD3 and play a similar role to STAT3 inhibitors and thus enhance the sensitivity of ATC to doxorubicin. In this study, we showed that combination of GSK-J4 and doxorubicin synergistically and significantly induces the apoptosis of human ATC cells through increasing caspase 3 level. The detailed molecular mechanism of synergistic therapy needs to be further studied.

In conclusion, we found that the JMJD3 inhibitor GSK-J4 significantly inhibited the proliferation of ATC. In KRAS-mutant cells (the Cal-62 cell line), the synergistic effect of GSK-J4 and doxorubicin was obvious at lower concentrations, and the effect was stronger than that seen in the BRAF-mutant cell lines (**Figure 5**). Our findings provide a new method for the systemic treatment of KRAS-mutant ATC.

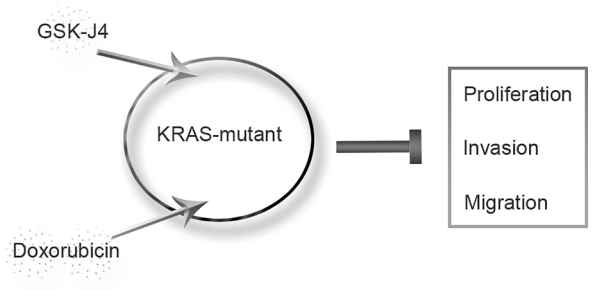

FIGURE 5 | Proposed model of KRAS-mutant anaplastic thyroid cancer with GSK-J4 and doxorubicin treatment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal laboratory of Zhongshan School of Medicine.

AUTHOR CONTRIBUTIONS

Equal contributors: BLi, I-YH, and BLu. Data collection and drafting: JL, WL, WZ, and BLi. *In vivo* experiments: I-YH and BLi. *In vitro* experiments: BLi and BLu. Statistical analysis: ZL and ZS. Manuscript polishing: BLi. Building figures: I-YH and BLu. Manuscript editing: JL and WL. Manuscript revision: BLi, BLu, YY and WZ. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00632/full#supplementary-material

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REVIEW
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Biomarkers for Pancreatic Neuroendocrine Neoplasms (PanNENs) Management—An Updated Review

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Pancreatic neuroendocrine tumors (PanNENs) are rare sporadic cancers or develop as part of hereditary syndromes. PanNENs can be both functioning and non-functioning based on whether they produce bioactive peptides. Some PanNENs are well differentiated while others—poorly. Symptoms, thus, depend on both oncological and hormonal causes. PanNEN diagnosis and treatment benefit from and in some instances are guided by biomarker monitoring. However, plasmatic monoanalytes are only suggestive of PanNEN pathological status and their positivity is typically followed by deepen diagnostic analyses through imaging techniques. There is a strong need for new biomarkers and follow-up modalities aimed to improve the outcome of PanNEN patients. Liquid biopsy follow-up, i.e., sequential analysis on tumor biomarkers in body fluids offers a great potential, that need to be substantiated by additional studies focusing on the specific markers and the timing of the analyses. This review provides the most updated panorama on PanNEN biomarkers.

Keywords: pancreatic tumor, pancreatic neuroendocrine tumor, biomarker, neuroendocrine syndrome, FDG (18F-fluorodeoxyglucose)-PET/CT

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INTRODUCTION

Neuroendocrine neoplasms (NENs) are rare and heterogeneous tumors of epithelial origin arising from cells of the neuroendocrine system. Pancreatic NENs (PanNENs) are low incidence diseases accounting for less than 3% of all pancreatic malignancies but their prevalence is relatively high and is actually rising (1). PanNEN patients account for 8.1% of total NEN cases (SEER 18) (2), present metastases at diagnosis in 60–80% of cases (3) and can be subgrouped in functioning (F-PanNENs) and non-functioning neoplasms (NF-PanNENs) depending on their ability to secrete active hormones associated with a specific symptomatology. They can occur as sporadic and isolated tumors or in the context of complex hereditary syndromes, such as multiple endocrine neoplasia type 1 (MEN1), von Hippel–Lindau disease (VHL), neurofibromatosis 1, and tuberous sclerosis (4–6). MEN1, in particular, is the commonest syndrome associated with PanNENs and about 10% of all PanNEN patients are affected by MEN1 syndrome (1, 7). PanNENs prognosis differs widely, with some tumors having an indolent nature, with a reasonable length of survival

even with a metastatic presentation and others being very aggressive with poor prognosis. PanNENs prognosis heterogeneity is in part recognized by the World Health Organization (WHO) classification system. Three independent PanNEN staging systems coexist and are suggested by the European Neuroendocrine Tumor Society (ENETS), the American Joint Committee on Cancer (AJCC) and the World Health Organization (WHO) respectively (2, 8–10). WHO classification is based on cellular proliferation (measured as mitotic count and Ki-67 expression; see **Table 1**). WHO has recently updated NENs classification whereby well-differentiated NENs are defined Neuroendocrine Tumors (NETs) regardless the grading. This has generated a novel subgroup of well-differentiated tumors with high Ki-67/mitotic index as G3 and poorly differentiated NENs defined as Neuroendocrine Carcinomas (NEC) which are G3 by definition (2, 8, 10). The ENETS staging system is based on TNM classification (1, 14) whereas the AJCC—draws on the TNM staging for pancreatic adenocarcinoma (5, 9); see **Table 1** for a comparison). Although the grade of disease is prognostic, several differences in the clinical behavior remain between each subgroup, making personalized treatment challenging for PanNENs. There is a clear unmet clinical need for novel prognostic and predictive biomarkers able to improve grading and staging assessments, guide prognostication and support treatment decisions. We will provide here a general overview of the existing and promising prognostic and predictive biomarkers for PanNENs.

PANCREATIC NENs BIOMARKERS

Correct diagnosis and accurate staging are of primary importance when treating cancer patients and the use of biomarkers is pivotal in this challenge. An ideal biomarker should display high sensitivity for the diagnosis of NENs, to predict tumor aggressiveness (prognostic biomarker) and/or response to treatment (predictive biomarker) (15). Since several factors impact NEN patients' survival, a multi-analyte approach, which takes into consideration clinical, biochemical, histological and molecular features of the disease is required (16). Several parameters correlate with the overall survival of NEN patients. They include tumor localization, size, grade and stage, vascularization, presence of necrotic tissue and the presence of metastases (17, 18). NEN diagnosis starts with the biochemical quantification of circulating analytes in the plasma and/or serum of patients. Neuroendocrine markers can be divided into two main groups: non-specific markers that are virtually produced by all NENs (19) and specific markers that are primarily produced by F-NENs (**Table 2**).

Pancreatic NENs Non–specific Biomarkers

Non-specific PanNEN biomarkers include chromogranin-A (CHGA), Neuron Specific Enolase (NSE), Pancreatic Polipeptide (PP), Human Chorionic Gonadotropin (HCG), and Alpha Fetoprotein (AFP) (**Table 2**, **Figure 1**). Biochemical evaluation of these analytes can be easily performed on serum/plasma fraction of patients with suspected NENs. Aberrant levels of such non-specific markers should drive further and deepen diagnostic tests (30).

Chromogranin-A (CHGA) is a glycoprotein secreted by neurons and neuroendocrine cells, which is a precursor of bioactive substances such as pancreastatin, catestatin and vastatins I and II (31). Despite all members of granin family can be secreted by neuroendocrine tumors, CHGA is the only one routinely used in clinical practice. The assay has a high sensitivity (32) and good specificity (19). Increased levels of CHGA can be detected both in plasma and serum with a good correlation, suggesting either measurement can provide reliable evaluations of circulating CHGA (33). Circulating CHGA has been reported to correlate with tumor progression (19), presence of metastases (34), tumor burden and response to treatment in NENs, including PanNENs. In fact, CHGA decrease in serum can be considered a surrogate marker for treatment efficacy (35). In contrast, despite two to three-fold increase of CHGA can be considered marker for NENs and also for neuroendocrine differentiation of other non-neuroendocrine cancers, several non-pathological factors, such as food intake (6) and several non-neoplastic endocrine diseases can increase its level in the bloodstream (36), making diagnosis challenging. For those patients affected by concomitant conditions, CHGA assay specificity may decrease up to 50%. Therefore, CHGA should be never considered a first-line diagnostic or screening tool in these sub-populations (37). Despite the above-mentioned limitations, up to now CHGA is the most used liquid biomarker not only in the diagnosis but also during the follow-up of NEN patients.

Neuron Specific Enolase (NSE) is an enzyme found in neurons and neuroendocrine cells, even if only 30–50%, of NENs secretes NSE (22, 32, 38). This marker may be elevated in 38–40% of high-grade GEP-NENs, including PanNENs thus providing also prognostic information (39). NSE levels have been directly associated with tumor differentiation, aggressiveness and size (39, 40) and it was found to inversely correlate with overall survival (OS) and with progression-free survival (PFS) in ENETS TNM stage IV. NSE has low sensibility but relatively high specificity (see **Table 2**). Indeed, NSE can be virtually overexpressed also by several non-neuroendocrine tumors, such as parathyroid cancer, prostate carcinoma, neuroblastoma, and it has been correlated with poor differentiation, prognosis and high-grade disease (24). For these reasons NSE alone is rarely used for diagnostic purposes or to distinguish NENs from non-endocrine tumors. Up to date, there is no robust evidence of the predictive role of NSE in predicting therapy efficacy and monitoring patients during follow-up. On the other hand, elevated baseline CHGA/NSE provide prognostic information on PFS and survival in patients with advanced PanNEN treated with the mTOR inhibitor everolimus (41). Evaluation of both NSE and CHGA concentration increases the reliability of NEN diagnosis; however, given the non-specific nature of these markers, they do not provide information on the primary tumor site and its origin (24).

Pancreatic Polypeptide (PP) PP is a 36 amino acid linear oligopeptide, primary secreted by the PP cells of Langerhans' islets (42). Despite its specific role is not well clarified it is

TABLE 1 | Current WHO grading guidelines and 8th AJCC/UICC—ENETS consensus for pancreatic neuroendocrine neoplasms (11, 12).

WHO 2017	G	Mitoses 10 HPF*	Ki-67 Index*	Genetic background	Neoplastic evolution•	8th AJCC/UICC—ENETS consensus for pan NET staging	
Well-differentiated NENs							
NET	G1	< 2	< 3	*ATRX*	G1-G2		
	G2	2-20	3 – 20	*MEN-1*	↓↓	T stage	
	G3	>20	> 20	*DAXX*	G3	T1	Confined to pancreas*, <2 cm
					↓	T2	Confined to pancreas*, 2-4 cm
Poorly-differentiated NENs					↓	T3	Confined to pancreas*, >4 or duodenum or bile duct invasion
NEC	G3	> 20	> 20	*RB* *Tp53*	NEC	T4	Invasion of vessels and contiguous organs
MiNEN**	G1-G3	NET/NEC + ADC/SCC		Mixed features			

*WHO, World Health Organization; NEN, Neuroendocrine Neoplasm; NET/C, Neuroendocrine Tumour/Carcinoma; HPF, Hight Power Field; AJCC, American Joint Committee on Cancer; ENETS, European Neuroendocrine Tumour Society; UICC, Union for International Cancer Control; ADC, Adenocarcinoma; SCC, Squamous cell Carcinoma. •Neoplastic evolution Current classification considers the possibility of an evolution with time of a well-differentiated G1-G2 NEN to a higher G3 and, even more rarely, toward a poorly differentiated NEC (13). **MiNENs (Mixed-NENs): may contain of non- neuroendocrine components (e.g., adeno or squamous) and neuroendocrine ones (at least 30% for each component) (11). *Specific parameters for PanNET according to 8th AJCC/UICC-ENETS consensus.*

supposed to regulate pancreatic, GI secretions (32) and hepatic glycogen levels (38). PP is generally considered a neuroendocrine differentiation marker with good specificity but low and variable sensitivity (30) (**Table 2**). Since 2015, PP has been suggested for the diagnosis of PanNENs (NCCN guidelines) (43) and ESMO 2012 consensus guidelines already considered PP diagnostic also for NF-PanNENs (29). Despite PP has been observed to be elevated in metastatic disease with increased sensitivity (up to 80%) (44), <50% of PanNEN patients presents with elevated serum PP (19). Additionally, serum concentrations of PP can be increased by many factors, including physical exercise, hypoglycemia, and food intake (32), as well as decreased by somatostatin and hyperglycemia, diarrhea, laxative abuse, increased age, GI inflammatory processes and chronic renal disease (45). Detection of high levels of circulating PP, together with CHGA is suggestive for PanNENs with increased sensitivity (30, 42). Production of PP and/or CHGA is observed in 100% of spontaneous and hereditary gastrinomas (46). In contrast, decline of PP level during patients monitoring is considered a good prognostic marker (19).

Finally, **human chorionic gonadotropin (HCG)** and **alpha-fetoprotein (AFP)** can be also considered in biochemical assessment of certain malignancies, although their use is limited (24). HCG is a glycoprotein physiologically synthesized by syncytiotrophoblastic cells of the placenta during pregnancy (24, 32) and it is composed of α and β subunits. The β subunit (β-HCG) is specific, since tumor cells usually lack the mechanism to link α and β subunits. An increased secretion of the β subunit is reported in pancreatic tumors and PanNENs. AFP is a peptide hormone produced during development. In adults increase of AFP in serum has been reported in NENs (25, 26). AFP-producing PanNENs are rare and often associated with other malignancies (47, 48). However, the literature is controversial on the sensitivity and specificity HCG and AFP, thus limiting their use in NENs (37, 49).

PanNENs Specific Biomarkers

Bioactive peptides retrieved in the blood of F-PanNEN patients are useful prognostic and predictive biomarkers (24). However, hormones are not always secreted and retrievable from the blood. Indeed, evaluation of expression directly on the neoplastic tissue is the gold standard for diagnosis. In addition, symptoms associated with their increased levels help both to diagnose and to identify the primary site of disease (50). F-PanNENs are named after the hormones they produce as insulinomas, glucagonomas, gastrinomas, somatostatinomas, VIPomas, which are suggestive of their cell-of-origin.

Circulating Biomarkers

Gastrin (GAS) is a linear peptide hormone secreted by G–cells of pyloric antrum, duodenum and pancreas implicated in the regulation of chloride acid release from parietal cells in the stomach, gastric motility and pancreatic secretion. A plasma concentration of GAS >300 pg/mL correlates with the presence of gastrinomas, even if GAS is secreted as well by functioning NENs especially in the context of MEN-1 and Zollinger–Ellison syndrome (ZES) (32).

Insulin (INS) is a dimeric peptide hormone of 51 amino acids, physiologically secreted by the β cells of the pancreatic islets in response to glycemia increase and

TABLE 2 | Biochemical biomarkers in use for PanNEN diagnosis, prognosis, and treatment monitoring.

Biochemical markers			Source	Level	Sens. (%)	Spec. (%)	Combinations improving sens./spec.	Clinical use	References
Non–specific	Chromogranin A	CHGA	Serum	63–14.750 ug/l	60–83	72–85	NSE; PP	For diagnosis and follow up in GEP-NENs and treatment monitoring	(20, 21) (22, 23)
	Neuron-specific enolase	NSE	Plasma	5–92 ug/l	33	73	CHGA	For diagnosis and follow up in GEP-NENs and treatment monitoring	(20, 21) (22, 23)
	Pancreatic-Polipetide	PP	Plasma	480–780 pg/ml	31-63	67	CHGA	For diagnosis and follow up in PanNENs	(23)
	Human Corionic Gonadotropin	HCG	Serum	Increased	na	Na	AFP; CHGA; PP; HCG	Indicative of pancreatic origin	(24)
	Alpha Fetoprotein	AFP	Serum	Increased	na	Na	HCG; CHGA; PP	Indicative of pancreatic origin and de-differentiation	(25, 26)
Specific	Gastrin	GAS	Serum	≥300 pg/mL	94	100	MEN-1; ZES	Diagnostic for Gastrinoma of pancreatic origin	(24, 27)
	Insulin	INS	Serum/ Plasma	≥43• pmol/L	52 - 94	92−100	Whipple's triad	Diagnostic for Insulinoma; suggesting for WD NETs.	(28)
	Glucagon	GCG	Plasma	500–1000 pg/mL	High	High	-	Diagnostic for Glucagonoma; suggesting for WD NETs; Indication for liver metastases	(24)
	Somatostatin	SST	Plasma	Increased°	na	Low	SSoma syndrome°	Diagnostic for SSoma of pancreatic origin;	(24)
	Vasoactive Intestinal Peptide	VIP	Serum/Plasma	75•−200 pg/dL	na	na	Verner Morrison	Diagnostic for ViPoma of pancreatic tail origin.	(29)

PanNENs, Pancreatic Neuroendocrine Neoplasia; GEP-NENs, Gastro-Entero-Pancreatic Neoplasia; WD NETs, well differentiated tumors; Sens., sensibility; Spec., specificity. •Diagnostic serum/plasma level in association with specific syndrome. °Somatostatin increase is very a-specific, increase SS level with SSoma syndrome is suggesting for GEP-NENs.

involved in the regulation of body anabolism. INS can increase as a consequence of several oncologic and non-oncologic conditions, therefore, its concentration alone does not represent a solid marker for insulinoma. Insulinoma should be suspected when patients display the so-called "Whipple's triad" symptoms: clinical evidence of hypoglycemia, serum glucose ≤40 mg/dL and improvement following administration of glucose (51).

Glucagon (GCG) is a peptide hormone secreted by pancreatic α-cells to increase catabolism thereby mobilizing energy reserves to free glucose molecules via gluconeogenesis and glycogenolysis. An increased plasma GCG level >500 pg/mL is indicative of glucagonoma albeit requires further diagnostic work-up to exclude non-oncologic reasons. By contrast, GCG levels >1000 pg/mL are diagnostic for glucagonoma and used in the clinical practice (52).

Somatostatin (SST) is a peptide hormone physiologically secreted by pancreatic δ-cells, APUD cells and gastric antrum D cells (53). SST can repress GCG and INS secretion by α and β cells of the pancreas, respectively. SST excess induces non-specific manifestations and it can result in the formation of gallstones, intolerance to fat in the diet, diarrhea and diabetes. Furthermore, increased levels of SST are not only associated with somatostatinoma of the pancreas but also with various extra-Pancreatic NENs (54). Hence, SST level *per se* is not sufficient to diagnose somatostatinoma but it requires very careful clinical assessment.

Vasoactive Intestinal Peptide (VIP) is a peptide hormone released by pancreatic and brain cells. It is both a neurotransmitter and a potent vasodilator regulating smooth muscle activity, epithelial cell secretion and blood flow in the gastrointestinal tract. VIPoma, a non-ß pancreatic islet cell

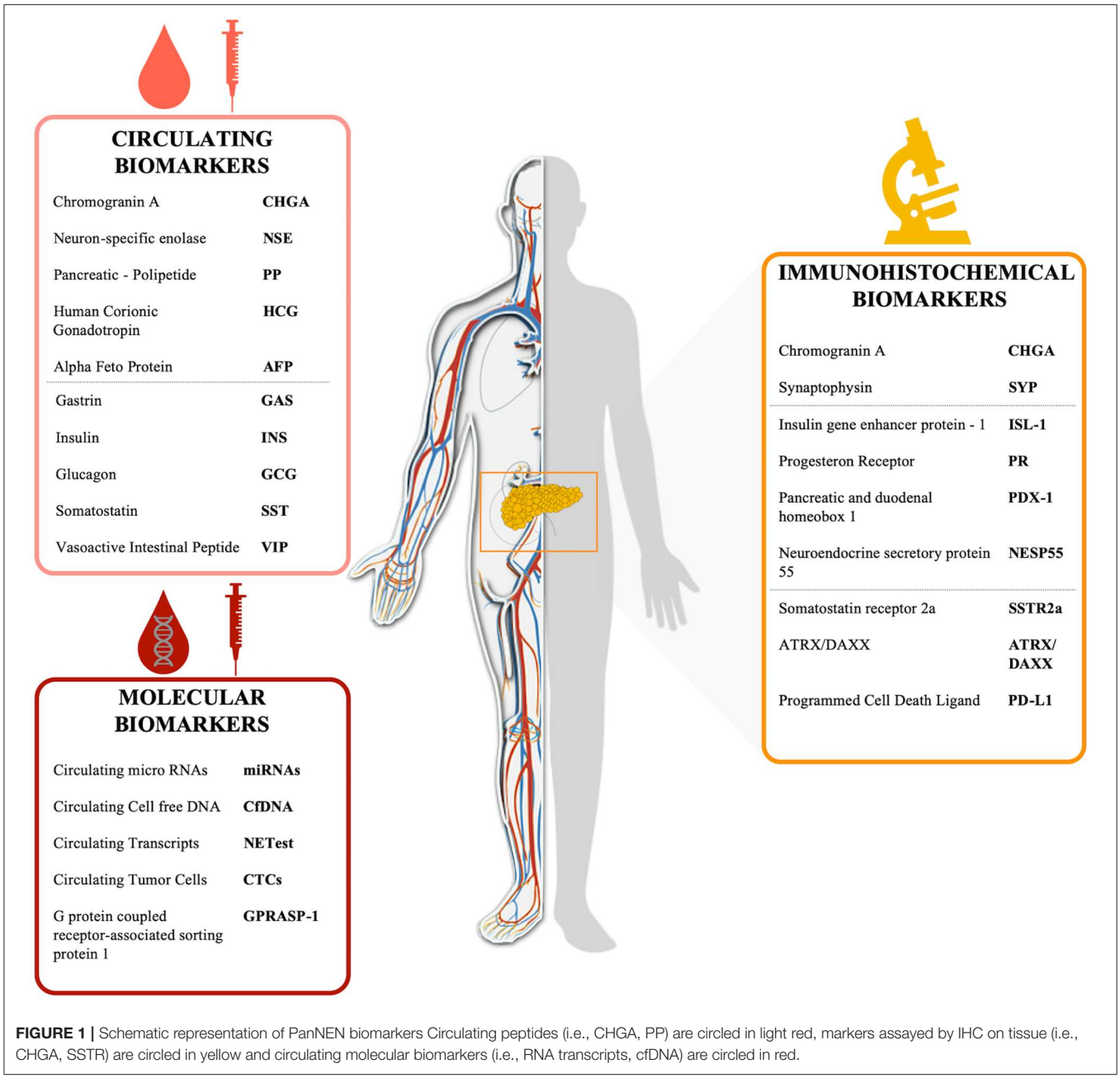

FIGURE 1 | Schematic representation of PanNEN biomarkers Circulating peptides (i.e., CHGA, PP) are circled in light red, markers assayed by IHC on tissue (i.e., CHGA, SSTR) are circled in yellow and circulating molecular biomarkers (i.e., RNA transcripts, cfDNA) are circled in red.

tumor, shows a syndrome of watery diarrhea, hypokalemia, and achlorhydria (WDHA syndrome) and it is diagnosed by a serum VIP concentration above 200 pg/dL. A mild increase in VIP concentration (75-200 pg/dL) can be also considered in patients with Verner Morrison syndrome (29). These biomarkers can be suggestive of a PanNEN. However, symptoms can often be nuanced or aspecific, and careful clinical and histo-pathological assessment remains mandatory.

Tissue Biomarkers

Histological diagnosis is usually assessed on surgical or endoscopic biopsies, on which morphological and marker distribution analysis is performed by immunohistochemistry (IHC) (29) (**Table 3**). PanNENs can also produce hormones that are not subsequently secreted, and specific stains for GAS, INS, and SST can confirm clinical symptoms without biochemical increase in serum. However, IHC alone for hormones and bioactive peptides cannot prove site of origin and confirm functionality of NENs (29). At present chromogranin A (CHGA) and synaptophysin (SYP) are considered the most specific markers for NEN differentiation by immunohistochemistry (62). CHGA is contained in the granules of neurons and pancreatic cells, it is a precursor of several functional peptide hormones such as vasostatins and pancreastatin. CHGA is widely expressed in well–differentiated NENs whereas generally low or focally positive in poorly–differentiated NEC (55). SYP is an integral

TABLE 3 | Immunohistochemical (IHC) biomarkers for PanNENs diagnosis, prognosis and treatment monitoring.

Immunohistochemical markers (IHC)			Source	Level	Combinations improving sens./spec.	Clinical use	References
Differentiation	Chromogranin A	CHGA	Surgical/endoscopic biopsy	Over-expressed	SYP	Diagnosis of NENs; Grading; Differentiation	(23) (24)
	Synaptophysin	SYP	Surgical/endoscopic biopsy	Over-expressed	CHGA	Diagnosis of GEP-NENs; grading; differentiation	(24)
Site of Origin	Insulin gene neanche homeeobox - 1	ISL-2	Surgical/endoscopic biopsy	Over-expressed in endocrine pancreas	Low expression in case of Gastrinoma	Over-expressed in Pan NENs (especially in WD tumors)	(55)
	Progesteron Receptor	PGR	Surgical/endoscopic biopsy	Positive	CHGA + SYP	Indicative of pancreatic origin (40-75%) (negative in GI-NENs)	(56)
	Pancreatic and duodenal homeobox 1	PDX-1	Surgical/endoscopic biopsy	Positive	CHGA + SYP	Indicative of pancreatic origin	(57)
	Neuroendocrine secretory protein 55	NESP55	Surgical/endoscopic biopsy	Focally positive	CHGA + SYP	Indicative of pancreatic origin (40–50%)	(56)
Prognostic/ Predictive	Somatostatin receptors 2a	SSTR2a	Surgical/endoscopic biopsy	Over-expressed	CHGA + SYP	Indicative of pancreatic origin; Predictive for PRRT treatment; inverse correlation with grading.	(58) (59)
	ATRX/DAXX	ATRX/ DAXX	Surgical/endoscopic biopsy	Loss of expression	CHGA + SYP	Prognostic for tumor aggressiveness; (associated with WD tumors)	(60)
	Programmed Cell Death Ligand	PD-L1	Surgical/endoscopic biopsy	Over-expressed	CHGA + SYP	Prognostic/Predictive for anti-PD-L1 therapeutic agents	(61)

PanNENs, Pancreatic Neuroendocrine Neoplasia; GEP-NENs, Gastro-Entero-Pancreatic Neoplasia; WD NETs, well differentiated tumors.

transmembrane glycoprotein expressed in neuroendocrine cells and neurons involved in synaptic transmission with a diffuse cytoplasmic immunostaining (63). CHGA and SYP combined assessment represents the first of a multi–step approach currently in use to confirm the neuroendocrine nature of the disease and then its pancreatic origin.

EMERGING MARKERS IN PanNEN

Tissue Biomarkers

Besides the validated diagnostic markers, other tissue biomarkers are under investigation to improve PanNENs management providing information on the site of origin, grading, immune and genetic landscape of the disease. In addition, novel biomarkers could be new therapeutic targets. Up to now several immunohistochemical panels have been proposed to identify primary tumor site of origin, especially in NENs of the gastro-entero-pancreatic (GEP-NENs) tract. Although many recent studies focused on these biomarkers they are not routinely used and validated for diagnosis and/or prognosis in PanNENs management.

Islet 1 (ISL-1) is a homeobox transcription factor expressed in all endocrine pancreatic cells (57). This pattern of expression suggests a general role in the development of multiple cell lineages of the endocrine pancreas. ISL-1 expression is detected in 70–82% of panNENs (64). Unfortunately, other GI–NENs, in particular NENs of the rectum, overexpress this marker (65) and gastrinomas of the pancreas show low expression of ISL-1 making its application as a general PanNEN diagnostic biomarker troublesome.

Progesteron Receptor (PR), represent a widely—studied, but still incoming and more specific pancreatic marker. Nuclear positivity for PR has been reported in most pancreatic endocrine tumors, and recent studies confirm PR expression in 40–75% of PanNENs (56, 64). In addition, PR immunoreactivity has been demonstrated to be strictly confined to endocrine compartment of normal and neoplastic human pancreatic islets (56, 64) and to be significantly associated with a favorable prognosis and a lower clinical stage (66). The relative expression of PR isoforms (PRA; PRB) have been reported to have a prognostic role in NENs from different site of origin (e.g., breast) (67, 68). Recent findings focused on the role of PRA and PRB in PanNENs demonstrated that PRB activation promotes Cyclin D1 (CCND1) overexpression and, as a consequence of c-Fos and c-Jun induction transcription factors supporting cell proliferation and tumorigenesis (69). In addition, progesterone signaling via PRA could inhibit tumorigenesis by PRB suppression. In addition, PRA can be a suitable predictive factor in PanNEN and inversely correlated with tumor progression (70).

Neuroendocrine secretory protein 55 (NESP55) is a protein belonging to the chromogranin family which can be considered

highly specific marker for PanNENs, since other GI-NENs subtypes show low to none expression of this protein (64). Recent findings report focal and specific expression of NESP55 in 40–74% of PanNENs in contrast with very rare expression observed in other GI-NENs and NENs of the lung and rectum (5 and 8%, respectively) (64, 71).

Paired box 8 (PAX 8) represents a transcription factor able to regulate organogenesis in a variety of organs (72). Although PAX8 has been considered a marker for renal development and neoplasms, Sangoi et al. observed high PAX8 reactivity in PanNENs and normal pancreatic islets in a large tissue microarray evaluation (73). In contrast with ileal or pulmonary NETs and NENs of duodenum, stomach, and rectum which were negative to PAX8 staining or show very low expression, respectively. PAX8 has been demonstrated to be particularly useful in metastatic NENs with unknown primary tumor site, the expression PAX8 in combination with ISL-1 could indicate pancreatic origin (5).

Pancreatic and duodenal homeobox 1 (PDX-1) is transcriptional activator of several genes, including insulin, somatostatin, glucokinase, islet amyloid polypeptide, and glucose transporter type 2 (74). PDX-1 immunoreactivity is reported in 54–100% of PanNENs (64). Despite PDX-1 can be expressed also by other GI-NENs, NENs of the ileum have been reported to be negative for PDX1 thus it can be useful, especially when used in combination with ISL-1, PAX8, and/or NESP55 in defining pancreatic site of origin when it is unclear. In addition, PDX-1 is involved in the early development of the pancreas and plays a key role in glucose-dependent regulation of insulin gene expression (74).

Among those, combinations of Islet 1 (ISL-1), Progesteron Receptor (PR), neuroendocrine secretory protein 55 (NESP55), paired box 8 (PAX8), and Pancreatic and duodenal homeobox 1 (PDX1) suggest pancreatic origin (73, 75–77). In addition, the well-known Somatostatin Receptors (SSTRs) and GLUT-1 are companion markers for imaging techniques which fulfill a primary role in PanNEN diagnosis and prognosis.

Somatostatin receptors 2a and 5 (SSTR2a and SSTR5) have been widely studied as prognostic and predictive biomarker in GEP-NENs since most of GEP-NENs shows diffuse SSTRs overexpression (78), especially G1 and G2 stage tumors (79). Indeed, an inverse correlation between SSTR2a expression and NENs differentiation has been observed (80). SSTR2a is particularly over-expressed in PanNETs compared to NENs of different origin (e.g., GI-NENs/NEC). SSTRs represent the molecular target for ^{68}Gallium-labeled compounds and PET/CT (^{68} Ga - PET/CT scan) that has recently become the gold standard for the diagnosis and management of these tumors. Recent study by Liverani et al. observed an inverse correlation between ^{68}Ga - PET/CT uptake and tumor differentiation in a small GEPNENs subsets (81). Therefore, SSTR2 can be considered for both diagnostic and therapeutic purposes. Intriguingly, SSTR2 is more expressed in primary PanNENs than in metastases (82), suggesting a novel additional role of SSTR2a in monitoring the tumor progression (79). Most of those biomarkers are not yet used in clinical practice. However, multianalyte combinations should show higher sensitivity and might be more effective than the current use of monoanalytes as shown in some studies (83, 84). Several peptides and growth factors have been explored as biomarkers for PanNENs to improve early diagnosis and follow-up of NENs, among these α-Internexin, Paraneoplastic antigen 2 (PNMA2) and X-linked inhibitor of apoptosis (XIAP) are emerging immunocytochemical markers.

Glucose transporter 1 (GLUT-1) is a uniporter protein that mediates the transport of glucose molecules through the cell membrane. GLUT-1 is observed to be overexpressed in several tumors, probably related with higher metabolism and cell growth (85). Several studies have shown association between GLUT-1 expression and tumor aggressiveness, poor prognosis and neuroendocrine differentiation in a number of carcinomas (86–88). Fujino, M. et al. investigated the prognostic role of GLUT-1 in G1/G2 PanNENs. GLUT-1 overexpression correlates with grading, Ki-67 mitotic index, vessel invasion, lymph node metastases and poor disease free survival rate (89). In addition, HIF-1α overexpression was observed in GLUT-1 positive cases, suggesting a HIF-1α dependent induction of GLUT-1 in hypoxic conditions (89). In addition, GLUT-1 over expression in NENs correlates with an increased uptake of 2-deoxy-2-[fluorine-18] fluoro-D-glucose and positivity in PET-CT (90). High ^{18}F-FDG uptake is a useful prognostic marker in PanNENs (91), thereby GLUT-1 expression may be a good surrogate prognostic marker for ^{18}F-FDG captation. Altogether those evidences suggest that GLUT-1 expression might be taken into consideration for PanNENs prognostic assessment. Since ^{18}F-FDG uptake by PanNENs is a valuable prognostic marker associated with important aspects of tumor metabolism it is becoming of paramount importance to find biomarkers that correlate with this status for longitudinal analyses in patients. In line with this observation, our preliminary data, presented at the 2019 ESMO meeting reported a prognostic miRNA signature associated with ^{18}F-FDG PET status in PanNENs (92).

Programmed Cell Death Ligand (PD-L1), a protein involved in the immune checkpoint, is recently observed to be strongly upregulated in G3 tumor patients both on tumor and infiltrating immune cells, resulting in poor T-cell-mediated tumor surveillance (93). Thus, PD-L1 expression may represent a predictive biomarker for GEP-NENs patients who may benefit from immunotherapy (94). Interestingly, it has been recently reported that DAXX and ATRX molecular alterations correlate with increased tumor-associated macrophage (TAMs) infiltration thereby with inferior Disease Specific Survival rates, suggesting TAMs as potential prognostic biomarkers and targets for immune-modulating therapies in PanNETs (61). Finally, latest publications and communications at international meetings propose novel tissue markers with diagnostic, prognostic and/or therapeutic markers for PanNENs, such as Delta-like protein 3 (DLL-3). Interestingly, PD-L isoform 2 (PD-L2) has been found significantly overexpressed ($p < 0.001$) in PanNENs compared to non-pancreatic NENs (e.g., lung) (95). The same study identified that PD-L2 inversely correlates with presence of tumor necrosis and with PD-L1 expression levels ($p < 0.03$).

DLL-3 is a member of the Notch ligand family that is aberrantly expressed on the cell surface of Small cell lung

cancer (SCLC), Merkel cell Carcinoma (MCC) (96) and other neuroendocrine tumor cells (96–99) making it an attractive therapeutic target in NECs as proposed at latest international conferences, including AACR (96) and ESMO 2019 (100) annual meetings (96) and tested in ongoing trials on SCLC (TAOHE, NCT0306181).

α-Internexin is a cytoskeleton protein involved in tumorigenesis and disease progression (101) and is overexpressed in nervous system cell but also in insulinomas (102). Its evaluation in tumor tissue specimens has been observed to be useful as monoanalyte to predict and monitor treatment efficacy in insulinomas (102, 103). Furthermore, combination of α-Internexin and Ki-67 mitotic index, as prognostic multianalytes tests, is observed to predict tumor aggressiveness in insulinomas (89, 104–107). Loss or reduced expression of α-internexin protein represents potential prognostic marker for non-insulinomas PanNENs in terms of overall survival (OS) (102).

Paraneoplastic antigen 2 (PNMA2) is a neuronal antigen identified as marker of neurological paraneoplastic syndromes (108). PNMA2 shows correlation with disease progression and recurrence free survival in PanNENs (109).

X-linked inhibitor of apoptosis (XIAP) suppresses apoptosis in cancer cells (110, 111). It is a prognostic factor in cancer patients. Despite its role in PanNENs is not well established it is overexpressed in neuroendocrine GI tract and can represent a potential target for therapies (112–114).

Novel forthcoming DNA/RNA markers are also studied. DNA/RNA markers usefulness is mainly explored in the bloodstream via non-invasive liquid biopsy. Nevertheless, detection, analysis, and data interpretation of liquid markers are challenging and still under development. For this reason, many studies explored the expression pattern of DNA/RNA markers and/or molecular mechanisms, such as alternative lengthening of telomeres (ALT), non-coding RNAs, and mutational patterns also and primarily on tumor tissue specimens.

ALT is a tissue DNA prognostic marker for NENs. In PanNENs, ALT was shown to correlate with inactivating mutations in ATRX/DAXX genes (115, 116). Despite the literature is controversial about it, ALT expression is associated with larger tumor size, grading, vascular/perineural invasion and metastasis (117, 118). In contrast, other studies have found association with prognosis (119, 120).

MicroRNAs (miRNAs) are 21-24 nucleotides non-coding RNAs (ncRNAs) that interfere with gene expression. A plethora of studies have been performed and propose specific tissue miRNA signatures to distinguish PanNENs patients from healthy individuals and the primary tumor from the metastatic disease with a prognostic and/or predictive role. For example, Roldo et al. described a tumor specific miRNA signature defined by miR-103 and miR-107 expression and by the absence of miR-155 expression distinguishing PanNEN from normal pancreatic tissue (121). Furthermore miR-204 is primarily expressed in insulinomas and correlates with insulin expression on tissue (122).

Genetic Alterations Promoting Nen Development

Before the last decade genetic studies on molecular alterations of GEP-NENs were limited and mainly based on data from genetic syndromes associated with endocrine neoplasms. The diffusion and fruition of next-generation sequencing and other high-throughput techniques (microarray expression, miRNAs, and methylome analyses) in recent years have provided a larger amount of genetic and epigenetic information and a wider view of these malignancies, and especially of PanNENs, from a genetic perspective as reviewed in a very comprehensive manner by several authors (119, 123–130).

This information improved patients' stratification. Indeed, the WHO 2017 update for PanNENs proposed the separation of PanNECs and PanNENs, based on molecular alterations and regardless of the grading (14, 131–133). *TP53* and *RB1* combined loss has been confirmed to be driver mutation of pancreatic carcinoma development. PanNECs represent the 7, 5% of all PanNENs (134) and they are characterized by *TP53* and *RB1* inactivating mutations 20–73 and 71%, respectively while NENs, including G3 NENs with higher Ki-67 percentage and proliferation index do not display these mutations (124, 125, 134–138). RB1 is a key negative regulator of the cell cycle via p16 and other proteins. Indeed, loss of p16 immunostaining has been reported in 20–44% PanNECs, alone or in combination with Rb loss (134, 139–142). Interestingly, RASSF1A, another cell cycle repressor of downstream to Rb displayed methylation of the promoter in 10–60% of PanNECs, pinpointing the crucial role of cell cycle deregulation in carcinomas tumorigenesis (143–146). Interestingly *TP53* inactivation and/or P53 protein nuclear accumulation have been identified in 20-70% and 65-100% of PanNECs respectively (134, 142, 147–149).

A specific mutational pattern has been also reported for PanNENs, that lack *RB/TP53* mutations or an impaired RB/P53 expression. These tumors frequently display *DAXX/ATRXX* (9–25%) and *MEN-1* (10–36%) mutations or protein impaired expression (150, 151). The first whole-exome study on PanNETs, identified *ATRX* and *DAXX* as mutated genes, located in the chromatin remodeling compartment (119). *ATRX/DAXX* loss occurs in 18 and 25% of PanNETs and leads to ALT phenomenon, chromosomal instability and higher tumor stage suggesting this mutation is a late event in the neoplastic transformation (116, 152, 153). A second effect of *ATRX/DAXX* alteration concerns PTEN and, as consequence the inhibition of the PI3K/mTOR pathway (117, 119, 154, 155).

In addition, whole-genome/exome studies identified *PTEN* and *TSC1/2* as potential driver mutations in NENs development when compared to carcinoma tumorigenesis, with a frequency of inactivating lesions among PanNEN cases of 7 and 6%, respectively (119, 156). These alterations, in particular *RB1/TP53* loss, are particularly important for diagnosis and prognosis to distinguish NECs from G3 PanNENs, especially in challenging cases as when morphology and immunostaining are unreliable (131, 132, 151, 157, 158).

Germline Mutations and Sporadic PanNEN Development

Genetic studies on molecular alteration of GEP-NENs has been limited and mainly based on data from genetic syndromes associated with endocrine neoplasm for a long time. Genetic syndromes with recurrent germline mutated genes such as *MEN*, *VHL*, *NF1*, and *TS* (159–164)have been demonstrated to favor GEP-NENs development in about 10% of all NENs (4). Interestingly, somatic mutations on the same genes have been reported to promote sporadic PanNEN onset, with variable frequencies. Data derived from hereditary syndromes first, and from sequencing of sporadic PanNENs later, highlighted the involvement of two main pathways in PanNENs development: cyclin-dependent cell cycle regulation (*MEN-1)* and the PI3K/mTOR pathway (*MEN-1, VHL, NF-1, TS*).

Multiple Endocrine Neoplasia type I is an autosomal dominant disease, promoting the development of pancreatic endocrine tumors in 60% of patients (165). It is caused by germline-inactivating mutations in the *MEN-1* gene (166, 167) and by subsequent somatic loss of the normal allele (168). *MEN-1* gene alteration has been also reported in 44% of sporadic NETs (127). For these reasons it is considered one of the main genes involved in NET biology (119, 156, 169–173). *MEN-1* loss affects a large number of cellular activities, including (a) histone methylation and expression of the *CDKN2C/CDKN1B* cell cycle inhibitors (174); (b) PI3K/mTOR signaling via Akt (175); (c) homologous recombination (HR) through interactions with DNA repair complexes (e.g., RAD51 and BRCA1)(176, 177). In addition, *MEN-1* mutations have been associated with loss of P27 as an early alteration in NET development (178).

Von Hippel–Lindau disease is caused by inactivating mutations of the *VHL* gene. *VHL* is observed to be inactivated also by deletion or methylation in up to 25% of sporadic PanNETs (127). *VHL* inactivation leads to the activation of the hypoxia induced pro-proliferative signaling (179, 180).

Neurofibromatosis type I disease derives from germline mutations of *NF1* that are associated with NEN development in 10% of patients affected by the syndrome. NF1 protein product is a negative regulator of PI3K/mTOR pathway which holds a key role in NEN tumorigenesis (169, 181). Nevertheless, *NF1* has been rarely reported to be mutated in sporadic PanNENs (127).

Inactivating mutations in *TS* lead to Tuberous Sclerosis Complex (TSC) syndrome and to sporadic PanNENs in 35% of cases (127). This is caused by inactivation of *TSC1* and *TSC2*, thus inhibiting PI3K/mTOR signaling downstream of AKT1 (119, 182).

Chromosomal and Epigenetic Alterations

Mutational events alone cannot be traced back and explain all cases of NEN. Evidence points instead to chromosomal and/or epigenetic alterations as origin of neuroendocrine transformation in about 50% of cases. CNV analysis and whole-genome sequencing (117, 124, 156) allowed the definition of four PanNENs subtypes based on chromosomal alterations: (i) loss of chromosome 11q (where *MEN1* resides); (ii) a recurrent pattern of whole chromosomal loss (RPCL) in association with higher mitotic index, ALT and *ATRX/DAXX* inactivation; (iii and iv) patterns of chromosome gaining, complementary to losses of the RPCL group and associated with higher risk of metastasis (126, 183–187). In addition, whole-genome mutational analysis, identified 10% of germline mutations in base-excision repair (*MUTYH*) and homologous recombination repair (*BRCA2, CHEK2*) genes (119, 182).

From a transcriptional perspective PanNENs have been classified into 3 subtypes, which are related to key pathways of NEN disease, namely, chromatin remodeling in *MEN1*-like tumors, PI3K/mTOR in insulinoma-like tumors and hypoxia-related genes in the metastasis-like primarytumors cluster (188).

DNA methylation alteration is also found and is associated with PanNETs. Hyper-methylation of RASSF1A, HIC-1, CDKN2A, VHL, and MGMT genes for example has been reported in a large fraction of PanNETs (189–191). In contrast, hypo-methylation was reported for ALU and LINE1. In particular LINE1 has been associated to poor prognosis and chromosomal instability in ATRX/DAXX negative tumors (190, 192, 193).

Liquid Forthcoming Markers in PanNENs

Three key methods allow a comprehensive assessment of the neuroendocrine disease: clinical evaluation, imaging, and biomarkers assessment (62, 84, 194). Imaging is complex, based on sophisticated and expensive technologies, and often fails to predict early changes of the disease and to anticipate progressions or resolve pseudo-progressions (195). In addition, standard serial CT/MRI imaging have well-described sensitivity limitations (196) and may even provide false negative output in comparison to functional imaging ^{68}Ga-somatostatin analogs (SSA)-PET/CT (197, 198). Furthermore, imaging can be invasive as it exposes patients to repetitive radiation sessions. Both clinical and imaging strategies, have high intra-observer variability and are operator-dependent (199). In contrast, blood biomarkers represent an easy-to-detect and non-invasive method to evaluate disease with objective measurements (62, 84). The advent of sophisticated and sensitive technologies has revolutionized the concept of biopsy, changing the focus from a tumor tissue-oriented framework to a systemic vision of the disease. Liquid biopsy allows the detection of specific nucleic acids in body fluids and it has particularly benefited from NGS and quantitative PCR approaches, partially overcoming the limit of tumor heterogeneity present in tissue biopsies (195, 200). Application of those analyses to blood samples has clear advantages, by allowing multiple and consecutive measurements to follow disease recurrence and clinical management outcomes. The National Institute of Health (NIH) has classified bio-markers into three categories for diagnosis and/or clinical applications (201): (i) Type 0 markers are 'indicators of the natural history of disease'. They can directly or indirectly correlate with diagnosis, prognosis, and outcome of the disease. (ii) Type I markers 'describe the effects of an intervention in accordance with the mechanism of action of the drug' and reflect the general efficacy of treatment through a specific mechanism. Finally, (iii) Type II markers can be used as surrogates for tumor functionality or clinical endpoints (e.g., PFS is often considered

for GEP-NENs) (194). In addition, regarding the blood based multianalyte tests (mRNA transcripts, i.e., NETest), the Food and Drug Administration provides guidelines for *in vitro* diagnostic (IVD) tools development. Indeed, FDA defines as IVD "any reagent, instrument, and/or system intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or Liquid biomarkers include circulating cell-free DNA (cfDNA), circulating tumor cells (CTCs), small-non-coding molecules, as microRNAs (miRNAs) or long non-coding RNAs (lncRNA), blood transcripts (e.g., NETest) and proteins (**Table 4**).

The role of cfDNA in PanNENs is debated. CNV analysis of circulating cfDNA mirrors the presence of tumor-specific genetic alterations of PanNEN cells (59). Nevertheless, the prognostic value of cfDNA harboring *RB1* and *TP53* mutations, typically found in NECs, has not met a consensus yet and it is still under investigation. Similarly genetic alterations affecting *ATRX/DAXX* and *MEN-1* recently found in a subgroup of PanNETs with poor prognosis are not detected yet in cfDNA with a prognostic role (60, 124, 204).

The prognostic significance of CTCs is uncertain and reports about them in NENs are conflicting. Indeed, some studies associate CTCs increase and bone metastasis in NENs (205), whereas others highlight CTCs low sensitivity for PanNENs (84). At present, the 2016 Delphic consensus on circulating biomarkers in NENs has defined CTCs as a non–reliable marker, due to technical limitations in evaluating their number and phenotype.

Circulating miRNAs are more stable than mRNAs in biofluids and are largely explored as prognostic and/or predictive biomarkers in NEN patients (58, 202). Accordingly, several studies have produced signatures of circulating miRNAs associated with PanNEN tissue expression although few reporting prognostic power in PanNENs. Among those miR-21, miR-642, miR-210, miR-196a, miR-96, miR-182, miR-183, and miR-200 are the best characterized (121, 206–208). In addition, a set of 10 miRNAs (miR-125a,−99a,−99b,−125b-1,-342,-130a,−132,−129-2, and−125b-2) has been found to distinguish PanNETs from NEC, whereas miR-204 over-expression resulted to cluster insulinomas (209). Moreover, mir-21 overexpression, which affects PI3K/mTOR pathway via PTEN, has been shown to correlate with higher Ki-67 percentage and liver metastasis in PanNENs (209). Another study reported overexpression of miR-196a as an independent predictor of earlier recurrence, also associated with grade, stage, and lymphatic spread at diagnosis (208). Interestingly, despite the paucity of available preclinical models for NET disease, a metastasis-like (MLP) murine miR-signature (miR-23b,−24-1,−24-2,−27b,−132,-137,−181a1, and−181a2) has been detected and interestingly, it has also found to be overexpressed in about 65% of human PanNETs (188).

LncRNAs can promote angiogenesis, metastasis, and tumor suppressors escape (210–213). The role of lncRNA in PanNENs remains poorly explored in detail yet and most studies investigate their correlation with MEN1 gene-encoding "menin" protein in PanNETs. Modali et al., describe lncRNA *Meg3* (maternally expressed gene) as tumor-suppressor in PanNEN cells. PanNENs which produce Menin can activate *Meg3*. *Meg3* downregulates *c-Met* affecting cell proliferation, migration and invasion in insulinoma. Indeed, *Meg3* and c-MET levels are described to be inversely correlated, both in MEN1-associated PanNENs and sporadic insulinomas. In a recently published paper, Ji et al. found a significant difference in lncRNA and mRNA expression between pNEN tumors and adjacent normal tissues (214).

Blood Transcripts (mRNA)—The NETest

The NETest is a PCR-based multianalyte test built on tissue and peripheral blood transcripts using a signature of 51 NETs-related genes (23, 215). This algorithmic multigene assay was designed and validated specifically for GEP and bronchopulmonary NET diseases (83, 203, 216). Recent studies showed that NETest serves as diagnostic tool in PanNENs, since it distinguishes NET disease from cancers of different site of origin or non-neoplastic

TABLE 4 | Circulating and tissue molecular biomarkers for PanNENs diagnosis, prognosis and treatment monitoring.

Molecular Markers			Source	Level	Clinical use	References
Potentially prognostic and/or predictive	Circulating Tumor Cells	CTCs	Serum/plasma	Increased	Related to the PFS and OS	(202)
	Circulating cell free DNA	cfDNA	Serum/plasma	Increased	Indicative of pancreatic tumor origin, correlates with primary tumors mutations (e.g., ATRX/DAXX)	(59)
	Circulating transcripts	NETest	Serum/plasma	Presence of NET "finger print" genes	Prognostic for tumor aggressiveness; predictive for treatment efficacy.	(203)
	MicroRNAs	miRNAs	Serum/plasma*	Up/down−regulated	Diagnostic for site of origin; prognostic and potentially predictive for treatment efficacy.	(58)

PanNENs, Pancreatic Neuroendocrine Neoplasia; PFS, progression-free survival; OS, overall survival. Serum/plasma: also detected in tumor and healthy tissue. Useful for correlation between circulating and primary tumor markers.*

conditions (e.g., chronic pancreatitis) with 94% accuracy. Indeed, the NETest resulted much more accurate than current validated CgA measurements, which displayed 56% overall accuracy (83).

NETest can act as both type 0 and type II biomarker, as it serves both as diagnostic tool and for prognostication on disease status (stable/progressive disease) and treatment efficacy prediction (154, 203, 216–219). Latest meta-analysis by Oberg et al. recently reported a diagnostic accuracy of NETest of 95–96% with a mean diagnostic odds ratio (DOR) of 5 853, positive likelihood ratio (+LR) of 195, and negative LR of 0.06 in determining the presence of neuroendocrine neoplasia (194). The normalized 51-marker signature is interrogated using 2 separate mathematical algorithmic analyses composing a single score, which is scaled 0–100% (the NETest score). The updated cut-off of NETest score for diagnosis is 20% (220–225). These data are consistent with the definition of IVD functional ability to establish a diagnosis and determine the presence/absence of the disease. In addition, the NETest was 84.5–85.5% accurate as a marker of disease status, distinguishing stable disease from progressive disease at the time of the blood draw (219–221, 224–226). These data show the highest (>80%) concordance with the current Response Evaluation Criteria in Solid Tumors (RECIST) among NET biomarkers, fulfilling NIH proposed cut-off (149). In addition, NETest is observed to be related to functional imaging (e.g., ^{68}Ga-somatostatin analogs (SSA)-PET/CT) with 98% concordance in GEP-NETs, including PanNETs (225). Further studies are required to assess whether a blood test can replace imaging for disease monitoring, thus limiting radiation exposure and potential healthcare costs reduction. NETest is also a valuable marker of natural history of the disease (type 0), with an accuracy of 91.5–97.8%. In particular, a cut-off of 40 has been demonstrated to distinguish stable disease (≤40%) and progressive disease (≥40%) (227). Finally, NETest can be considered also an interventional/response biomarker with 93.7–97.4% accuracy, fulfilling type II biomarker requirements of NIH classification. In particular, a decrease and/or stabilization (≤40%) of NETest levels correlates with response to PRRT; in contrast with increased levels (≥40%) during therapy and/or follow up which is suggestive of treatment failure (219, 224, 226–228). To enforce NETest clinical value as a PRRT—response biomarker, it can be combined with PRRT Predictive Quotient (PPQ) to improve patient stratification (228). PPQ is a blood-based classifier based on specific variants of the NETest gene signature (encompassing growth factor signaling and metabolomic gene expression) (154, 228–230). PPQ has been demonstrated to predict tumor response to internal radiations in broncopulmonary and GEP-NETs (231). PPQ—positive score can predict PRRT-responders with ∼95% accuracy (227). Modlin et al. recently observed that NETest levels significantly decrease after PRRT treatment PPQ positive cohort of "responders," in contrast with increased level of NETest reported in PPQ-negative cohort of "non-reponders." NETtest levels negatively correlate with PPQ positivity ($p < 0.0001$) (229, 230). Additionally it has been recenty shown that NETest: (i) high levels (≥40) better predict disease recurrence in post-operative PanNETs alone (AUC: 0.82) or in combination with RECIST criteria (88% accuracy) (232); (ii) is very accurate also for GEP and broncopulmonary NEN with 100% diagnostic accuracy for the latter (233) and (iii) decreased levels after radical resection provide early assessment of surgical efficacy (234).

Very recently, G protein coupled receptor-associated sorting protein-1 (GPRASP-1), known as lysosomal sorting and Beclin2 regulator, has also been proposed as a novel circulating biomarker for neuroendocrine differentiation for PanNENs (235) (**Table 5**).

CONCLUSIONS

Currently available biomarkers for PanNENs have limitations and this unmet need hampers early diagnosis, prognosis and follow-up, stratification of patients for therapy selection and post-operative recurrence identification. Assessment of monoanalytes (e.g., CHGA, SYP) is poorly informative about the pathological status and positivity always need to be supported by further investigations. However, the combination of markers, as CHGA/PP, CHGA/NSE, GLUT-1/Ki-67 have been shown to increase specificity and sensitivity, to trace back to the primary tumor site and to better assess the disease aggressiveness, thus helping clinicians in therapeutic decisions. Liquid biopsy represents the new frontier for PanNEN diagnosis and prognosis, since the sensitivity of technologies is constantly increasing,

TABLE 5 | Novel potential biomarkers for PanNENs diagnosis, prognosis and treatment monitoring.

Putative markers			Source	Level	Clinical use	References
Potentially Prognostic and/or Predictive	Delta-like protein 3	DLL-3	Surgical/endoscopic biopsy	Over-expressed	Potentially prognostic and therapeutic target	(236, 237)
	Tumor-Associated—Macrophages	TAMs	Surgical/endoscopic biopsy	Increased	Associated to reduced DSS	(61)
	G protein coupled receptor-associated sorting protein 1	GPRASP-1	Serum	Down-regulated	Neuroendocrine de-differentiation	(235)
	Glucose transporter 1	GLUT-1	Surgical/endoscopic biopsy	Over-expressed	Prognostic for higher metabolism and tumor aggressiveness	(90)

PanNENs, Pancreatic Neuroendocrine Neoplasia; GEP-NENs, Gastro-Entero-Pancreatic Neoplasia.

hence allowing the detection of smaller and smaller amounts of biomarkers with non-invasive procedures. This is leading to earlier diagnosis and more accurate assessment of minimal residual disease after treatment. However, the role of markers such as cfDNA and CTCs is still controversial and requires expensive equipment and well-trained personnel for the analyses. Conversely, the detection of non-coding RNAs, such as miRNAs and lncRNAs is less expensive and more accessible from an economical and a know-how stand-point. Notably, circulating RNAs can not only function as prognostic and/or predictive biomarkers, but also serve as therapeutic targets for tailored approaches, including miRNA replacement. Recently designed clinical trial, SENECA study (NCT03387592) and translational ones as the NET-SEQ study (NCT02586844) and the Royal Marsden PaC-MAn Study (NCT03840460) are at the forefront of this challenge. In particular the Italian SENECA trial focuses on some specific biomarkers on primary tumor tissues and for miRNAs on blood samples while NET-SEQ and PaC-MAN studies are investigating the molecular alterations in intestinal and pancreatic neuroendocrine tumors both in tissue and blood samples. Both studies leverage on NGS sensitivity to discover novel DNA/RNA-based biomarkers from liquid biopsies of NEN patients. We believe those trials will pioneer the identification of the next generation biomarkers for PanNENs.

AUTHOR CONTRIBUTIONS

MB wrote the review, prepared figures. FN helped in preparing the figures. SS, AB, and TI provided supervision on the topic. GS edited and commented on the manuscript. IG edited the manuscript. MM conceived and wrote the review.

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Extraskeletal Myxoid Chondrosarcoma: Clinical and Molecular Characteristics and Outcomes of Patients Treated at Two Institutions

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Background: Extraskeletal myxoid chondrosarcoma (EMC) is a rare subtype of STS, which usually arises in extremities. It carries reciprocal translocations involving the NR4A3 gene. It displays an indolent behavior, but studies with long follow-up showed a high proportion of local and distant recurrences. For patients with progressing metastatic disease anthracycline-based chemotherapy is the standard front-line regimen, though has limited activity. There is some evidence on possible activity of antiangiogenetics.

Methods: This is a retrospective study conducted at Istituto Oncologico Veneto and at Institut Gustave Roussy. All patients with a confirmed diagnosis of EMC from January 1980 to December 2018 were extracted from a prospectively maintained database.

Results: 59 patients were identified, 37 male (62.7%) and 22 female (37.3%) with a M/F ratio of 1.7/1. We performed molecular analysis in 23 cases, all carried a EWSR1-NR4A3. Out of 49 patients treated with curative intent, 28.6% developed local recurrence and 40.8% patients developed metastases. In patients who had been radically resected (R0) local recurrence occurred in 7.6% of cases and metastases occurred in 15.4% of cases; in patients treated with R1 surgery, rates of relapse were higher. Twenty patients received chemotherapy for metastatic disease; best response was partial response with clinical benefit in 50% of patients. Fourteen patients received a second line of chemotherapy, with 46.1% disease control rate. A drug holiday was proposed to 8 patients with a mean duration of 22.8 months. Median overall survival was 180 months for the study population and 76 months for metastatic patients. No significant prognostic role was found for all studied variables, yet a trend of better survival for complete surgery, location in extremities of primary tumor and solitary lung metastases was observed. Chemotherapy for metastatic disease was negatively associated with survival.

Conclusion: In this large retrospective cohort of patients with ECM, location of primary tumor and solitary lung metastases seem to be associated with better survival. Chemotherapy did not impact survival in unselected patients. Further research is necessary in order to identify more active regimens and to provide clinical and molecular factors to select patients that could delay systemic treatment for metastatic disease.

Keywords: extraskeletal myxoid chondrosarcoma, anthracycline, NR4A3, drug holiday, chemotherapy (CHT), surgery, trabectedin

INTRODUCTION

Extraskeletal myxoid chondrosarcoma (EMC) is a very rare sarcoma subtype, which usually arises in the extremities, although it can originate from any anatomic site, and despite the name suggesting a soft-tissue-only location, there are reports of primary ECM of the bone (1–3). First diagnosis occurs commonly in middle age, with a wide age range, and it is more frequent in men than in women (1, 2, 4–6). It is currently classified by the World Health Organization classification under the category of tumors of uncertain differentiation (7).

EMC was first recognized in 1953 by Stout and Verner, but it was only in 1972 that Enzinger defined precisely its clinical and pathologic features (8). Importantly, ECM harbors recurrent genetic rearrangements involving the *NR4A3* gene on chromosome 9, representing an extremely useful confirmatory diagnostic clue (9) *NR4A3* fuses with different partners. The most frequent is *EWSR1* (EWS RNA-binding protein (1) followed by *TAF15* [TAF 15 RNA polymerase II, TATA box binding protein (TBP)-associated factor]. Rare fusion transcripts have been described, which are TCF12-NR4A3, TFG-NR4A3, and HSPA8-NR4A3 (4, 5, 10, 11).

EMC is considered a disease with an indolent behavior characterized by slow growth, but studies with adequately long follow-up point at a high proportion of local and distant recurrences. In retrospective series, the extension of surgery appears to affect the recurrence rate (1).

Metastases more frequently occur in lung, followed by bone, lymph nodes, and soft tissue. Despite a high rate of metastases, patients typically exhibit remarkably high survival rates of approximately over 80% at 5 years and over 60% at 10 years (1, 2, 6).

At present, no predictive factor is available to help decision-making for metastatic disease, and in particular to define whether systemic treatment should be used. Standard anthracycline-based chemotherapy, which is commonly used in the first-line treatment of advanced soft tissue sarcoma, has limited activity in this sarcoma subtype with variable reported response rates (12). There is some evidence on the role of anti-angiogenics in EMC. An Italian study reported activity of sunitinib on 10 patients treated with sunitinib at the dose of 37.5 mg/day, with best response being partial response in 6 patients and stable disease in 2 patients (13). More recently a multicenter phase 2 study tested the activity of pazopanib, with partial response observed in 4 out of 22 eligible patients (18%) and stable disease in 16 patients (73%) (14).

METHODS

All consecutive patients with a confirmed diagnosis of EMC treated at Istituto Oncologico Veneto in Padova and at Institut Gustave Roussy in Villejuif from January 1980 to December 2018 were extracted from a prospectively maintained database. Electronic health records were reviewed and the following data were collected: date of diagnosis, age at diagnosis, pathology report, performance status, site of primary tumor, site of metastases, first treatment approach, quality of surgery, local recurrence, data on radiation therapy, response to treatment, and survival. Response to treatment was evaluated by means of RECIST criteria, version 1.1 for all patients with metastatic measurable disease. The clinical outcome of each patient was recorded as alive or dead as of November 30, 2019.

Overall survival was measured from different time points (from first diagnosis, from the diagnosis of metastatic disease, and from the start of chemotherapy) to date of death; patients lost at follow-up were censored at last follow-up visit. Statistical analysis was carried out with R version 3.6.1. Survival was estimated with the Kaplan–Meier product-limit method; comparisons between groups were performed using the log-rank test.

The analysis for NR4A3 transcript was performed with qRT-PCR assay: total RNA was extracted from 10 sections of FFPE tissue with manual RNeasy FFPE Kit (Qiagen) and was quantified using a spectrophotometer. cDNAs were synthesized from 1,000 ng of RNA by a reverse transcription, using two reverse primers, respectively, in the exon 2 and in the exon 3 of NR4A3 gene. Three microliters of each cDNA was used in a real-time PCR assay. Primers and probes used in this assay are specific for the detection of the following fusion: EWSR1(ex7)/NR4A3(ex2), EWSR1(ex12)/NR4A3(ex3), EWSR1(ex13)/NR4A3(ex3), and TAF15(ex6)/NR4A3(ex3).

RESULTS

Patients' Characteristics

A total of 59 patients were identified, 37 were male (62.7%) and 22 were female (37.3%) with a male-to-female ratio of 1.7/1. Median age at diagnosis was 54 years (range, 24–90 years). Patients' characteristics are described in **Table 1**.

Primary tumor site was lower limbs in 40 patients (67.8%), abdominal wall in 7 patients (12%), upper limbs in 6 patients (10%), chest in 3 patients (5.1%), and other sites in 3 patients (i.e.,

TABLE 1 | Patients' characteristics.

Patients characteristics	N. (%)
Male	37 (62.7%)
Female	22 (37.3%)
Age	24–90 years
Primary Location	(n. 59)
lower limb	40 (67.8%)
upper limb	6 (10%)
chest	3 (5.1%)
abdomen	7 (12%)
other	3 (5.1%)
Metastatic Sites	(n. 26)
Lung	23 (88%)
Bone	4 (15.4%)
Other	14 (53.8%)
Primary Treatment	(n. 59)
Surgery	42 (71.2%)
Chemotherapy	10 (16.9%)
Radiation therapy	3 (5%)
NA	4 (6.9%)
Extension of Surgery	(n. 49)
R0	26 (53%)
R1	12 (24.5%)
R2	2 (4%)
NA	9 (18.5%)
First Line of Chemotherapy	(n. 20)
Anthracycline-based	11 (55%)
Oral cyclophosphamide	4 (20%)
Other regimens	5 (25%)
Second Line of Chemotherapy	(n. 14)
Anthracycline-based	3 (21.4%)
Trabectedin	3 (21.4%)
Other regimens	8 (57.2%)
Locoregional Treatment	
Radiation therapy	23 (48.9%)
Lung metastasectomy	8 (17%)
Excision of local recurrence	14 (29.8%)
Radiofrequency	2 (4.3%)

vulva, heart) (5.1%). Location was not available in four patients (6.9%). Median tumor size was 10 cm (range, 1.5–25 cm).

Molecular analysis was performed in 23 patients, detecting the presence of *EWSR1–NR4A3* fusion in all cases.

Fifty-three patients presented with localized disease and six patients were metastatic at diagnosis. Out of 49 patients treated with curative intent, 20 patients (40.8%) developed metastases and 14 patients (28.6%) developed local recurrence.

Most frequent metastatic site was the lung (22 patients); 4 patients had bone metastases and 12 patients presented metastases in other sites (lymph nodes, soft tissue).

Treatment Description

The first treatment was surgery for 42 patients, chemotherapy for 10 patients, and radiation therapy for 3 patients. Of the patients treated with chemotherapy, seven received treatment in the pre-operative setting.

TABLE 2 | Outcome of surgery.

Type of resection	N. (%) Total	N. (%) Local recurrence	N. (%) Metastases
R0	26	2 (7.6%)	4 (15.2%)
R1	12	5 (41.6%)	7 (58.3%)
R2	2	NA	NA

Data on extension surgery were available for 40 patients: 26 had radical (R0) surgery, 12 patients had surgery with microscopic margin infiltration (R1), and 2 patients had macroscopic presence of tumor (R2).

Among patients with R0 surgery, two had local recurrence (7.6%) and four developed metastatic disease (15.4%); among patients with R1 surgery, five had local recurrence (41.6%) and seven developed metastatic disease (58.3%); of the two patients with R2 resection, one was metastatic and did not undergo further surgery, and the other one did not experience local recurrence after re-excision and radiation therapy. Outcome of surgery is described in **Table 2**.

Twenty patients received chemotherapy for metastatic disease, with 11 patients receiving an anthracycline-based regimen (4 patients received doxorubicin alone and 7 patients received a combination regimen). For 10 evaluable patients treated in first-line setting, best response was partial response in one case and stable disease in five cases with an overall control rate of 60%. Four patients received oral cyclophosphamide obtaining stable disease as best response in one case (control rate 25%); five patients received other regimens (i.e., etoposide, trabectedin), with two patients experiencing stable disease and two patients experiencing progression as best response. Control rate with chemotherapy of all assessable patients was 50%.

Fourteen patients received second-line chemotherapy, which, in three cases, was an anthracycline-based regimen, and all experienced progressive disease as best response; three patients received trabectedin, with two patients experiencing stable disease as best response; pazopanib was administered to one patient with stable disease as best response; other regimens (i.e., etoposide, cyclophosphamide) were used in seven cases, obtaining stable disease in two patients and progression in all other patients as best response. Control rate with second-line chemotherapy in assessable patients was 46.1%.

Among the 20 patients treated in the first-line setting, 6 patients were treated in pre-targeted therapy period (i.e., trabectedin; anti-angiogenics). Data on systemic treatment are reported in **Table 3**.

Seventeen patients received loco-regional therapy, which was radiation therapy in 23 cases, pulmonary metastasectomy in 8 cases, excision of local recurrence in 14 cases, and radiofrequency ablation in 2 cases, with a wide range of number of treatments per patients (from 1 to 17).

A drug holiday was proposed to eight patients with a mean duration of the therapeutic break of 22.8 months (range, 2–41

TABLE 3 | Disease control-rate with of chemotherapy.

Line of Treatment	N. (%)	Control Rate
First-Line	20	50%
Anthracycline-based	11	60%
Oral cyclophosphamide	4	25%
Other regimens	5	50%
Second-Line	14	46.1%
Anthracycline-based	3	0%
Trabectedin	3	66%
Other regimens	7	28.5%
Pazopanib	1	100%

months), and with two patients still being observed at the time of the writing of this manuscript.

Survival Analysis

Out of 59 patients, data for 4 patients were not available either because they were seen just once for second opinion or because they were lost at follow-up. With a median follow-up time of 72 months, 20 patients have died.

For the entire group of patients, median OS (mOS) was 180 months, with 75% of patients being alive at 5 years and 63% of patients being alive at 10 years. Considering only patients with metastatic disease, median OS was 76 months.

Median time from diagnosis to metastatic disease was 5.9 years with a proportion of 40.8% of patients treated with curative intent developing metastatic disease.

Extension of primary surgery seemed to impact overall survival, with patients with R0 surgery having a trend toward better survival than patients with R1 and R2 surgery, as shown in **Figure 1**. The presence of local recurrence did not affect survival ($p = 0.54$).

No difference in survival rates was observed according to gender (mOS not achieved for female vs. 136 months for male patients $p = 0.409$) as shown in **Figure 2**.

Overall survival for patients with primary location in extremities seemed to be better when compared to other primary sites, yet no statistical difference was observed (mOS 180 vs. 73 months; $p = 0.250$) as shown in **Figure 3**.

Location of metastases in the lung trended toward better survival compared to location in other sites, yet again no statistical difference was observed (mOS for lung metastases not reached, mOS for patients with lung and other sites' metastases being 73 months, and mOS for patients with metastases only in extrapulmonary sites 62 months, $p = 0.137$) as shown in **Figure 4**.

Chemotherapy in patients with metastatic disease was significantly associated with worse survival (mOS 72 vs. 81 months, $p = 0.009$). Median progression-free survival for patients receiving first-line chemotherapy was 9 months. No predictive role of the studied variables was observed for progression-free survival.

Univariate analysis showed metastases other than lung or mixed, and administration of chemotherapy for metastatic disease being associated with worse survival, with metastatic sites retaining prognostic significance as independent risk factor for survival in multivariate analysis (**Table 4**).

No difference in survival was observed between patients being treated for metastatic disease before targeted therapy era and those treated after (mOS 72 months vs. mOS NA, $p = 0.59$).

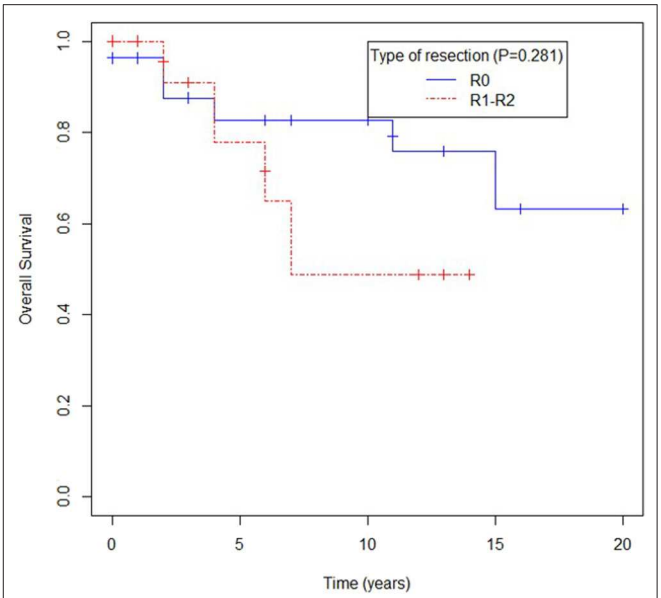

FIGURE 1 | Overall survival according to extention of primary resection.

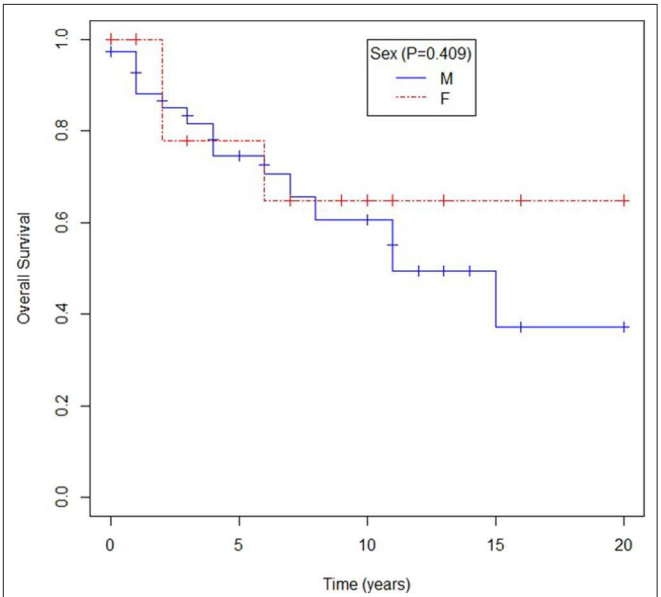

FIGURE 2 | Overall survival according to sex.

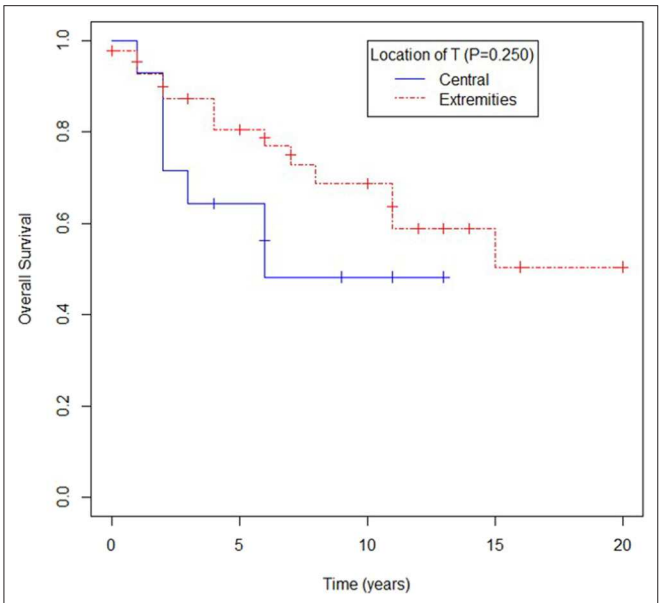

FIGURE 3 | Overall survival according to location of primary tumor (T): central (visceral, trunk, head and neck) or extremities.

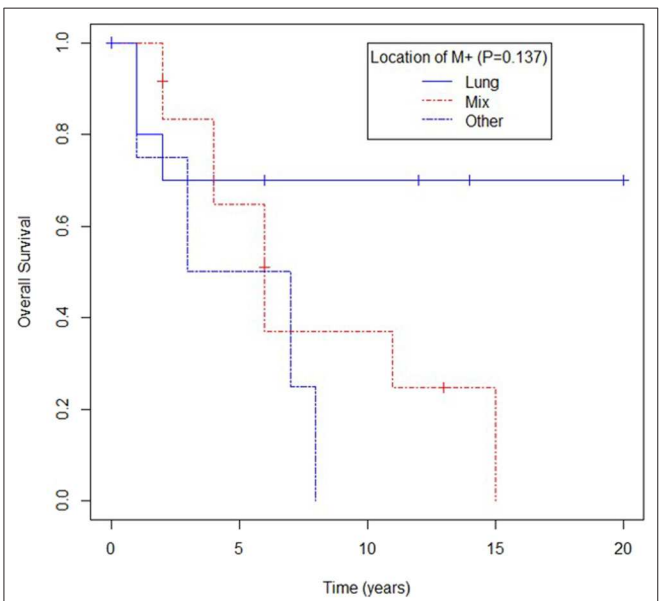

FIGURE 4 | Overall survival according to location of metastases (M) in lung, mixed (lung and other) or other.

TABLE 4 | Univariate Cox analysis for risk factors.

	Univariate Cox regression analysis	
Characteristic	**HR (95% CI)**	***p*-value**
Gender		
Male	Reference	
Female	0.686 (0.263–1.786)	0.440
Age		
For each 1-year increase	1.009 (0.979–1.041)	0.554
Primary location		
Central	Reference	
Extremities	0.567 (0.222–1.446)	0.235
Location of metastases		
None	Reference	
Lung	2.856 (0.572–14.250)	0.201
Mixed	6.665 (1.779–24.970)	**0.005**
Other	11.431 (2.465–53.000)	**0.002**
Type of resection		
R0	Reference	
R1-R2	2.021 (0.540–7.570)	0.296
Palliative chemotherapy		
No	Reference	
Yes	3.856 (1.349–11.020)	**0.012**

HR, hazard ratio; CI, confidence interval. p value significance are indicated in bold..

DISCUSSION

This study provides to date one of the largest series of an ultra-rare soft tissue sarcoma subtype with molecular data.

Consistent with previous reports, this study showed a predominance of the disease in male patients. EMC can occur at any age, but most patients in reported series are in the fifth and sixth decade (1, 2, 5, 6), as in our series in which median age at diagnosis is 56 years.

Our study also confirms the higher incidence of primary location in lower limb, which was observed in previous reports, where the most frequent site is thigh followed by upper arm and girdles, with only 10% of tumors arising in chest wall, abdomen, or other sites (1, 2, 4, 5, 15).

As for the role of surgery, there is extensive literature showing that incomplete or marginal surgery in patients with soft tissue sarcoma is associated with high rate of recurrence and metastases (16, 17). In a retrospective series of 117 patients with EMC treated with surgery as primary intent, a rate of 48% of local recurrence and 46% of metastatic recurrence was reported, with extension of surgery not identified as independent risk factor (18). In another surgical series on 87 patients, with data of quality of surgery available for 43 patients, a higher rate of local and metastatic recurrence was observed for patients receiving marginal surgery (1). Data from our study showed a correlation between extension of surgery and rate of local and metastatic recurrence and also a trend of better survival for patients receiving R0 surgery, in accordance to overall data for unselected histological type of soft tissue sarcoma (19–21).

Our study confirms that EMC's behavior is that of an indolent tumor, with most patients having very long survival rates even in the presence of metastatic disease.

Though the presence of distant metastases is an independent adverse risk factor, in our study, we identified patients with solely

lung metastases as a subgroup with a better survival at univariate and multivariate analysis, with a proportion of 70% of patients alive at 10 years (1, 15, 18).

Survival rates for primary tumors with central localization were slightly worse than other primary sites, reinforcing evidence deriving from other EMC retrospective series [18] and from a large case series that analyzed causes of death in patients with low-grade sarcomas (22).

As for the role of chemotherapy in advanced disease, in our study, standard anthracycline-based chemotherapy was not associated with better survival and, on the contrary, use of chemotherapy appeared to be associated with worse survival. Indeed, anthracycline-based regimens when used as first-line treatment showed a disease control rate of 60%, which is consistent with a previous retrospective study (12) and showed little or no benefit in second-line treatment, with no data on responses ever reported in further lines for this ultra-rare histotype. Other regimens used in first line showed lower control rate, with an overall control rate from other chemotherapy regimens in the range of 50% with no complete responses and low rates of partial response, confirming data of literature (1). Due to the retrospective nature of the study, no definitive conclusion can be drawn on the role of chemotherapy, and the negative impact of chemotherapy on survival could as well be biased by a higher likelihood to propose chemotherapy to patients with higher tumor burden or who are highly symptomatic, therefore having a worse prognosis independent of chemotherapy.

Trabectedin used as second-line regimen achieved a disease control rate of 66%; this is consistent with the only data reported of use of Trabectedin in EMC to date. In a subgroup of patients with diagnosis of EMC treated in the phase II trial of trabectedin, two patients achieved stable disease as best response (23). Filannino et al. (24) described a good response to trabectedin associated to radiation therapy showing synergy.

In our study, two patients received an angiogenesis inhibitor in second line and third line of treatment; both achieved stable disease as best response.

In our study, we observed a progression-free survival time of 9 months, which is higher than what was reported by Drillon et al. in 2008 in 21 patients (5.2 months) and consistent with data reported in 2013 on the use of anthracyclines in 11 patients in the series by Stacchiotti et al. (12, 14) (8 months), but shorter than median progression-free survival achieved with Pazopanib in a recent phase II trial that enrolled 23 patients (19 months) (1).

Again, given the limitations of retrospective data, data on progression-free survival time can be biased by different timing of restaging scans.

Of note, our study is the first to our knowledge to provide data on drug holiday, with long intervals of chemotherapy-free time for eight patients (mean duration of drug-free interval 22.8 months), suggesting the safety of such practice.

No analysis could be made to take into account the type of molecular alteration given the fact that all patients carried an EWSR1-NR3A4 translocated EMC.

CONCLUSION

Our study provides clinical and molecular data from one of the largest series of an ultrarare soft tissue sarcoma subtype.

Our data could not suggest any definitive role for quality of surgery of primary tumor, though radical surgery is associated to lower rates of local and metastatic relapse, while showing that location of primary tumor and solitary lung metastases can be prognostic for better survival.

Furthermore, our study adds evidence to the poor performance of anthracycline-based chemotherapy, which was not associated with better outcomes, yet the use of trabectedin translated in overall fair disease control rates.

Our data also suggest the safety of including drug holidays in the treatment strategy of metastatic disease.

Further research is necessary in order to identify more active regimens and to provide clinical and molecular factors to select patients that could delay or even avoid systemic treatment for metastatic disease.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico of Istituto Oncologico Veneto. The patients/participants provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BC drafting the work, substantial contributions to the acquisition, analysis, and interpretation of data. AL, MR, MM, ML, RC, PD, SI, MS, PT, PR, AD, CR, and VZ substantial contributions to the acquisition, analysis, and interpretation of data. AB revising the work critically for important intellectual content, contributions to the acquisition, analysis, and interpretation of data.

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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.

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Insulin-Like Growth Factor 2 mRNA-Binding Protein 3 Modulates Aggressiveness of Ewing Sarcoma by Regulating the CD164-CXCR4 Axis

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Ewing sarcoma (EWS) is the second most common bone and soft tissue-associated malignancy in children and young adults. It is driven by the fusion oncogene EWS/FLI1 and characterized by rapid growth and early metastasis. We have previously discovered that the mRNA binding protein IGF2BP3 constitutes an important biomarker for EWS as high expression of IGF2BP3 in primary tumors predicts poor prognosis of EWS patients. We additionally demonstrated that IGF2BP3 enhances anchorage-independent growth and migration of EWS cells suggesting that IGF2BP3 might work as molecular driver and predictor of EWS progression. The aim of this study was to further define the role of IGF2BP3 in EWS progression. We demonstrated that high *IGF2BP3* mRNA expression levels correlated with EWS metastasis and disease progression in well-characterized EWS tumor specimens. EWS tumors with high *IGF2BP3* levels were characterized by a specific gene signature enriched in chemokine-mediated signaling pathways. We also discovered that IGF2BP3 regulated the expression of CXCR4 through CD164. Significantly, CD164 and CXCR4 colocalized at the plasma membrane of EWS cells upon CXCL12 stimulation. We further demonstrated that IGF2BP3, CD164, and CXCR4 expression levels correlated in clinical samples and the IGF2BP3/CD164/CXCR4 signaling pathway promoted motility of EWS cells in response to CXCL12 and under hypoxia conditions. The data presented identified CD164 and CXCR4 as novel IGF2BP3 downstream functional effectors indicating that the IGF2BP3/CD164/CXCR4 oncogenic axis may work as critical modulator of EWS aggressiveness. In addition, IGF2BP3, CD164, and CXCR4 expression levels may constitute a novel biomarker panel predictive of EWS progression.

Keywords: IGF2BP3, metastases, CD164, CXCR4, Ewing sarcoma

INTRODUCTION

Ewing sarcoma (EWS) is a rare disease but it is still the second most common malignancy of bone and soft-tissues affecting pediatric age. It is characterized by a very aggressive behavior, high propensity for metastasis, specifically to bone and lung. Metastases occur in 30–40% of patients with localized disease, while 20–25% of patients present metastasis at diagnosis. The current standard treatment of EWS is a multimodal approach consisting of surgery and/or radiotherapy, and a multiagent chemotherapy, which confers a 5-years survival rate of 70% in patients with localized tumor. On the contrary, metastatic disease has a survival rate of 30%, independently of intensification of chemotherapeutic regimens (1). The identification of novel therapeutic strategies and reliable predictors of patient survival is therefore imperative to improve the outcome for metastatic patients.

While the genetic features of EWS are well-defined (2), as well as the contribution of the fusion gene *EWS-FLI1* to oncogenesis (3), the molecular mechanisms underlying EWS metastases are still poorly understood (4, 5).

EWS is characterized by one of the lowest mutation rates among all tumors (6–8) and this genomic stability is conserved in metastasis (9). On the contrary, epigenetic heterogeneity is prevalent in EWS, and even increased in the metastatic stage (10–12).

In general, EWS metastatic progression is regulated by multifactorial mechanisms, which include the dynamic activation of stress-adaptive or cellular plasticity pathways mediated by epigenetic or post-transcriptional mechanisms (5, 13–16). Previous reports have shown that EWS cells increase the expression of genes associated with metastasis, such as *CXCR4* or *HIF-1α*, through post-translational histone modifications or RNA binding proteins (RBPs) activity (15, 17, 18). The G protein-coupled receptor chemokine (C-X-C motif) receptor 4 (CXCR4), activated by its natural ligand CXCL12, promotes migration of EWS cells (13, 15).

RBPs, along with microRNAs and long non-coding RNAs, represent major post-transcriptional regulators of gene expression, due to their ability to bind RNA sequences and finely tune nuclear export, translation/degradation rate, and intracellular localization of their multiple transcript targets (19).

We have recently identified insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3) as a major determinant of EWS aggressiveness (20, 21). IGF2BP3 has a critical role in modulating multiple mRNAs, thereby regulating tumor initiation and progression (22). Accordingly, IGF2BP3 has recently emerged as putative prognostic biomarker for several tumors, including leukemia, carcinomas, and sarcomas (23).

In this study, we initially discovered that IGF2BP3 is significantly upregulated in metastatic lesions of EWS patients as compared to primary tumors, prompting us to investigate the molecular contribution of this RBP to the migration and dissemination of EWS cells. We then identified for the first time an oncogenic axis consisting of IGF2BP3/CD164 and CXCR4, which confers migratory advantage to EWS cells, particularly under stress-adaptive conditions.

TABLE 1 | Clinical-pathological features of primary localized EWS patients included in the study.

Characteristics		qRT-PCR (*N* = 48)		Microarray (*N* = 29)		IHC (*N* = 50)	
		No	**%**	**No**	**%**	**No**	**%**
Gender	*Female*	11	22.9	10	34.4	15	30
	Male	37	77.1	19	65.5	35	70
Age	*≤14 years*	22	45.8	10	34.4	14	28
	>14 years	26	54.2	19	65.6	36	72
Location	*Extremity*	33	68.7	22	75.8	47	94
	Central	4	8.3	2	6.9	3	6
	Pelvis	11	23	5	17.2	0	0
Surgery	*YES*	38	79.2	20	68.9	46	92
	NO	10	20.8	9	31	4	8
Local Treatment	*RxT*	10	20.8	9	31.0	4	8
	RxT+Surgery	11	23	5	17.2	8	16
	Surgery	27	56.2	15	51.7	38	76
Response to chemotherapy*	*Good*	10	26.3	5	25	15	32.6
	Poor	28	73.7	15	75	31	67.4

**Data available for 38 patients in qRT-PCR, for 20 patients in microarray and for 46 cases in IHC.*

qRT-PCR, quantitative Real-Time PCR; IHC, immunohistochemistry, RxT, radiotherapy, EWS, Ewing sarcoma.

MATERIALS AND METHODS

Clinical Specimens

This study included EWS specimens from primary localized tumors and EWS metastatic lesions. EWS diagnosis and treatment were performed at the IRCCS Istituto Ortopedico Rizzoli (Bologna, ITALY). For diagnosis, histological, immunohistochemical, and molecular features were considered (24). For therapy, patients underwent local treatment (surgery and/or radiation therapy) and systemic induction chemotherapy. All the patients included in this study were enrolled in previously approved prospective studies (25, 26). For those patients who underwent surgery, histologic response to chemotherapy was examined in accordance to Picci et al. (27). Clinical-pathological features of EWS patients, updated to 2018, are summarized in **Table 1**.

Cell Lines

For *in vitro* studies, the following patient-derived EWS cell lines were employed: A673 cells were provided by Dr. H. Kovar (St. Anna Kinderkrebsforschung, Vienna Austria) while TC-71 cells were provided by T.J. Triche (Children's Hospital, Los Angeles, CA). Cell lines authentication was executed by short tandem repeat (STR) polymerase chain reaction (PCR) analysis using a PowerPlex ESX Fast System kit (Promega, Madison, WI, USA) and the last control was performed in December 2017. Absence of mycoplasm contamination was assessed every 3 months using MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland). Stable silencing of IGF2BP3 was achieved using short hairpin RNA (shRNA; TRCN0000074673) included in a pLKO.1 vector,

and subsequent selection in puromycin (2 μg/ml; Sigma, St. Louis, MO, USA), as previously described (20, 21). Cell lines were cultured as previously reported (28). For hypoxia studies, cells were cultured in 1% O_2 using a Galaxy 14S incubator (New Brunswick, Eppendorf, Milano, ITALY) at 37°C and 5% CO_2.

Transient silencing of CD164 was performed using short interfering RNA (siRNA) from GE Healthcare Dharmacon (Lafayette, CO, USA); SMART POOL siGENOME_siRNA (M-016196-00-0020). As control, siGENOME_non-targeting siRNA was employed (D-001206-13-05). siRNAs (80 nM) were transfected into EWS cells using TransIT-X2 (Mirus, Madison, WI, USA) in accordance with the manufacturers' protocol.

RNA-seq and Bioinformatics Analyses

RNA extraction, cDNA libraries, sequencing, reads alignment, and normalization were performed as previously described (21). Hierarchical supervised clustering was performed using GeneSpring 11.02 software on differentially expressed genes using Pearson's correlation. Enrichment analysis of differentially expressed genes was performed using MetaCore software (GeneGo, Thomson Reuters).

Gene Expression Analysis

Extraction of total RNA from snap-frozen tissue samples, human mesenchymal stem cell (hMSC) primary cultures, and EWS cell lines was carried out using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Quantity and quality of obtained RNA were measured by NanoDrop (NanoDrop ND1000, ThermoFisher Scientific, Waltham, MA, USA) and/or by electrophoresis analysis. Reverse transcription was performed using High Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Obtained cDNA was amplified by quantitative Real-Time PCR (qRT-PCR) in a ViiATM 7 Real-Time PCR System (Applied Biosystems). Predesigned TaqMan probe (Applied Biosystem) was employed for *IGF2BP3* (Hs00559907_g1) expression level measurement. Primers set for *CD164 (*Fw: 5′-GAGTGCTGTAGGATTAATTGGA AAAT-3′, Rv: 5′-GGGAGGAATGGAATTCTGC-3′), *CXCR4* (Fw:5′-ACGCCACCAACAGTCAGAG-3′, Rv: 5′-AGTCG GGAATAGTCAGCAG-3′), and *Nanog* (Fw: 5′-CCTATGCCT GTGATTTGTGG-3′, Rv: 5′-GATCCATGGAGGAAGGA AGA-3′) were employed for SYBR green quantization. Primer pairs for *GAPDH*, used as a reference gene, were employed as reported previously (29). RT^2 Profiler Cancer Inflammation and Immunity Crosstalk PCR Array, profiling 84 genes involved in those pathways, was purchased from Qiagen (Hilden, Germany). Relative expression of analyzed transcripts was quantified following the $2^{-\Delta\Delta Ct}$ method (30).

Immunohistochemistry

Paraffin-embedded EWS specimens were incorporated in tissue microarrays (TMAs) and processed for immunohistochemistry (IHC) using an avidin–biotin–peroxidase method (Vector Laboratories, Inc., Burlingame, CA, USA). An overnight incubation with the following primary antibodies was performed: anti-CD164 (sc-271179, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:50, anti-CXCR4 (ab2074, Abcam, Cambridge, UK) diluted 1:50, anti-IGF2BP3 (sc-47893, Santa Cruz Biotechnology) diluted 1:50. Samples were classified as follows: negative, when no staining was observed; positive when weak, moderate, or strong staining was observed.

Western Blotting

For western blotting analysis, cells were harvested, rinsed with PBS and lysed with ice-cold lysis buffer (50 mM TrisHCl pH = 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM sodium fluoride, protease, and phosphatase inhibitors). Western blotting was performed according to standard procedures. Membranes were incubated overnight with the following primary antibodies: anti-IGF2BP3 (RN009P, dilution 1:20000, MBL International, Woburn, MA, USA), anti-CXCR4 (ab124824, dilution 1:1000, Abcam), anti-CD164 (AF5790, dilution 1:1000, R&D Systems, Minneapolis, MN, USA), anti-HIF-1α (sc-10790, dilution 1:2000, Santa Cruz Biotechnology), and anti-GAPDH (sc-25778, dilution 1:10000, Santa Cruz Biotechnology). The following secondary antibodies were used: anti-rabbit (NA934) and anti-mouse (NA9310V, GE Healthcare, Little Chalfont, UK) or anti-sheep (HAF016, R&D Systems) antibodies conjugated to horseradish peroxidase.

Motility Assay

Migration capability of EWS cells was established using Trans-well chambers (CoStar, Cambridge, MA, USA). 1×10^5 cells diluted in IMDM plus 1% FBS were seeded in the upper compartment, whereas IMDM plus 1% FBS and CXCL12 (100 ng/ml, ab9798, Abcam) were placed in the lower compartment of the chamber. After an overnight incubation, under normoxia or hypoxia, migrated cells were fixed in methanol. Cells were subsequently stained with Giemsa and counted.

Confocal Microscopy

Cells seeded on fibronectin-coated coverslips (Sigma) were serum starved for 24 h and pretreated with 80 μM dynasore (S8047, Selleckchem, Houston, TX, USA), or DMSO as control, in 1% FBS medium for 30 min at 37°C. Cells were then stimulated with CXCL12 (100 ng/ml, Abcam) in 1% FBS medium for 5 min at 37°C. Cells were fixed in 4% paraformaldehyde, permeabilized in Triton X-100 0.15%-PBS, blocked in 4% BSA and incubated with the following primary antibodies: anti-CXCR4 (ab124824, dilution 1:100, Abcam); anti-CD164 (sc-271179, dilution 1:50,Santa Cruz Biotechnologies). Anti-rabbit rhodamine (#31686, dilution 1:100, Thermo Scientific) and anti-mouse FITC (#31569, dilution 1:100, Thermo Scientific) were employed as secondary antibodies. Nuclei were counterstained with Hoechst 33256 (Sigma). Confocal analysis was performed using Nikon A1R confocal microscope with a Plan Apo 60x/NA 1.4 DIC N2 objective (Nikon, Minato, Tokyo, JP). To determine colocalization of the proteins of interest, Z-stacks were acquired at 0.25 μm intervals using the following settings: 1,024 × 1,024 pixel, 2 scanner zoom, 0.5 μm scan speed. Images were analyzed using Nis Elements AR4.20.01 software (Nikon, Minato, Tokyo, JP). Colocalization

was quantified by Mander's Colocalization Coefficient as we previously performed (31).

Ribo-Immunoprecipitation (RIP) Assay

The RiboCluster Profiler RIP-Assay kit (MBL International, Woburn, MA, USA) was used to identify IGF2BP3/transcript interactions, according to the manufacturers' protocol. For immunoprecipitation, anti-IGF2BP3 antibody (MBL International) or normal IgG (MBL International), used as a negative control, were used. Obtained RNA was reverse transcribed and qRT-PCR on equivalent amounts of cDNA was performed.

Statistical Methods

Differences among means were tested using a one-way ANOVA, if more than two groups were present, or Student's *t*-test. Spearman's rank test was employed to establish correlation between continuous variables. Spearman's

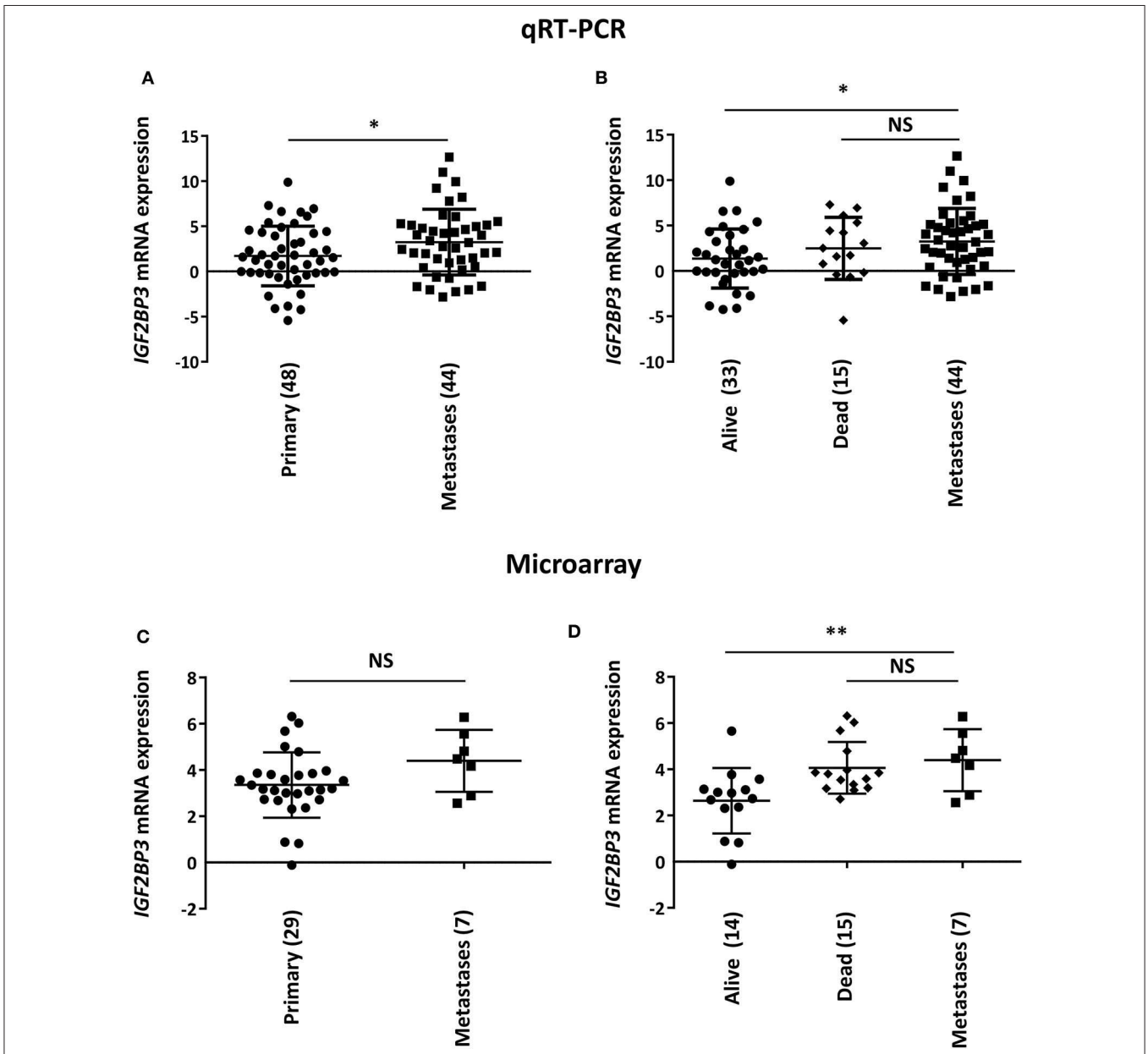

FIGURE 1 | Correlation between *IGF2BP3* and metastatic disease in EWS patients. Scatter plot analysis of *IGF2BP3* mRNA levels determined via **(A,B)** qRT-PCR or **(C,D)** microarray (GSE12102) in primary or metastatic EWS lesions. Differential expression between the groups was determined using **(A,C)** Student's *t*-test or **(B,D)** one-way ANOVA with respect to metastases. Mean ± *SD* of relative mRNA expression reported as $\log_2$ is shown. Human mesenchymal stem cells were used as calibrator. Number of analyzed cases is reported below each plot. $^*p < 0.05$; $^{**}p < 0.01$. NS, not significant.

correlation coefficients (r) were defined as weak ($0.1 < r < 0.39$), moderate ($0.4 < r < 0.69$), or strong ($0.7 < r < 0.89$), based on published definitions (32). Chi-square test was employed to establish correlation between categorized variables. Two-sided $p < 0.05$ was considered statistically significant.

RESULTS

IGF2BP3 Is Associated With Metastasis Formation and With Chemokine Signaling

To initially explore a possible correlation between IGF2BP3 and EWS metastasis, we measured *IGF2BP3* mRNA expression levels in 44 metastatic EWS lesions using as controls 48 primary untreated tumors from patients with localized disease at diagnosis (**Table 1**). Metastatic specimens displayed significantly higher expression of *IGF2BP3* mRNA as evaluated by qPCR (**Figure 1A**).

Notably, when the subset of primary tumors was divided according to overall survival of patients (alive vs. dead from disease), we found that the significant difference of *IGF2BP3* mRNA expression levels was only maintained when compared to tumors derived from patients with favorable overall outcome (alive; **Figure 1B**). This observation was confirmed in a different set of tumors previously analyzed by microarray analysis (29 primary tumors vs. 7 metastasis; **Table 1**) (9, 21). We did not detect a significant difference in *IGF2BP3* expression levels between primary and metastatic tumors (**Figure 1C**). However, *IGF2BP3* expression levels were upregulated in metastatic lesions as in primary tumors of patients dead from disease but they were significantly lower in primary tumors of patients who did not experience any recurrence or were alive at 10 years from diagnosis (**Figure 1D**).

To further define whether IGF2BP3-regulated mechanisms might have clinical impact, we took advantage of another set of 14 tumors analyzed by RNAseq (21). We compared the genetic expression profile of three primary localized EWS cases with the highest expression of *IGF2BP3* to three primary localized EWS cases with the lowest, if any, expression of *IGF2BP3* and identified a signature of 814 differentially expressed genes (615 upregulated and 199 downregulated, $P < 0.05$;

TABLE 2 | Enrichment analysis performed on 814 differentially expressed genes identified via RNAseq analyses in IGF2BP3-high vs. IGF2BP3-low expressers primary localized EWS cases using GeneGo annotation.

#	Pathway maps	Total	*p*-value	FDR	In data	Network objects from active data
1	*Immune response_ Antigen presentation by MHC class I: cross-presentation*	99	4.4E−20	5.7E−17	30	IRAP, Rab-3B, Syk, Cathepsin L, HSP70, Dectin-1, Fc gamma RI, C1q, Fc epsilon RI gamma, IP-30, TIM-3, Adipophilin, SREC-I, Cathepsin S, MSR1, MANR, FCGR3A, Rab-35, DAP12, TLR4, Rab-32, OLR1, TLR7, CD74, Cathepsin B, TLR2, gp91-phox, p67-phox, VAMP8, Fc gamma RII alpha
2	*Chemokines in inflammation in adipose tissue and liver in obesity, type 2 diabetes and metabolic syndrome X*	48	1.3E−19	8.5E−17	22	ITGA4, ITGAX, ITGAM, ICAM1, IL-1 beta, CCL2, MIP-1-alpha, Fc gamma RI, PLAUR (uPAR), MANR, IL-8, FCGR3A, MHC class II, VCAM1, TLR4, CD86, CD68, **CXCR4**, CD163, CD45, TLR2, CD14
3	*Macrophage and dendritic cell phenotype shift in cancer*	100	8.6E−14	3.7E−11	24	ITGAM, Activin A, PGE2R2, c-Rel (NF-kB subunit), IL-1 beta, EPAS1, PGE2R4, ILT4, IDO1, DLL1, MSR1, MHC class II, WNT5A, M-CSF receptor, TLR4, TLR7, CD86, GM-CSF receptor, Gas6, ILT3, IRF5, TLR2, SHIP, CSF1
4	*Rheumatoid arthritis (general schema)*	50	7.6E−13	2.4E−10	17	IL-15, IL-18, ICAM1, MHC class II beta chain, IL-1 beta, Fc gamma RI, HLA-DRB, TNF-R2, HLA-DRB1, FCGR3A, MHC class II, VCAM1, TLR4, CD86, TLR2, CD4, CSF1
5	*Neutrophil chemotaxis in asthma*	38	1.4E−12	3.5E−10	15	C5aR, GRO-2, CCL2, MIP-1-alpha, HSP70, PI3K reg class IB (p101), IL-8, PTAFR, GRO-3, CCR1, G-protein alpha-i family, GRO-1, TLR2, PI3K cat class IB (p110-gamma), ENA-78
6	*Immune response_ Antigen presentation by MHC class II*	118	4.1E−12	8.1E−10	24	MHC class II alpha chain, Syk, Cathepsin L, MHC class II beta chain, Dectin-1, Fc gamma RII beta, Fc epsilon RI gamma, IP-30, HCLS1, Cathepsin S, MANR, HLA-DM, Cathepsin V, FCGR3A, MYO1E, MHC class II, TLR4, CLEC10A, OLR1, Legumain, CD74, TLR2, CD4, SWAP-70
7	*Basophil migration in asthma*	55	4.4E−12	8.1E−10	17	CCL18, ITGAM, C5aR, ICAM1, FPRL2, CCL2, MIP-1-alpha, PLAUR (uPAR), PI3K reg class IB (p101), IL-8, CCR1, G-protein alpha-i family, VCAM1, GM-CSF receptor, PLAU (UPA), PI3K cat class IB (p110-gamma), CCL13
8	*Immune response_Alternative complement pathway*	53	2.9E−11	4.6E−09	16	C5aR, C3a, C3, C5 convertase (C3b2Bb), Factor I, Factor Ba, C3b, CRIg, Factor Bb, C3aR, C3 convertase (C3bBb), iC3b, C3dg, Factor B, C3c, Clusterin
9	*Maturation and migration of dendritic cells in skin sensitization*	41	7.8E−11	1.1E−08	14	MHC class II alpha chain, ICAM1, MHC class II beta chain, IL-1 beta, MEKK1(MAP3K1), HLA-DRB, TNF-R2, HLA-DRB1, IL-8, HLA-DRB3, MHC class II, HLA-DRA1, CD86, HLA-DRB5
10	*Cell adhesion_Integrin inside-out signaling in neutrophils*	77	1.8E−10	2.3E−08	18	Syk, ICAM1, Fc gamma RI, Cytohesin1, PI3K reg class IB (p101), IL-8, PTAFR, Lyn, Btk, DAP12, G-protein alpha-i family, Hck, GRO-1, Slp76, PI3K cat class IB (p110-gamma), IP3 receptor, FYB1, PREL1

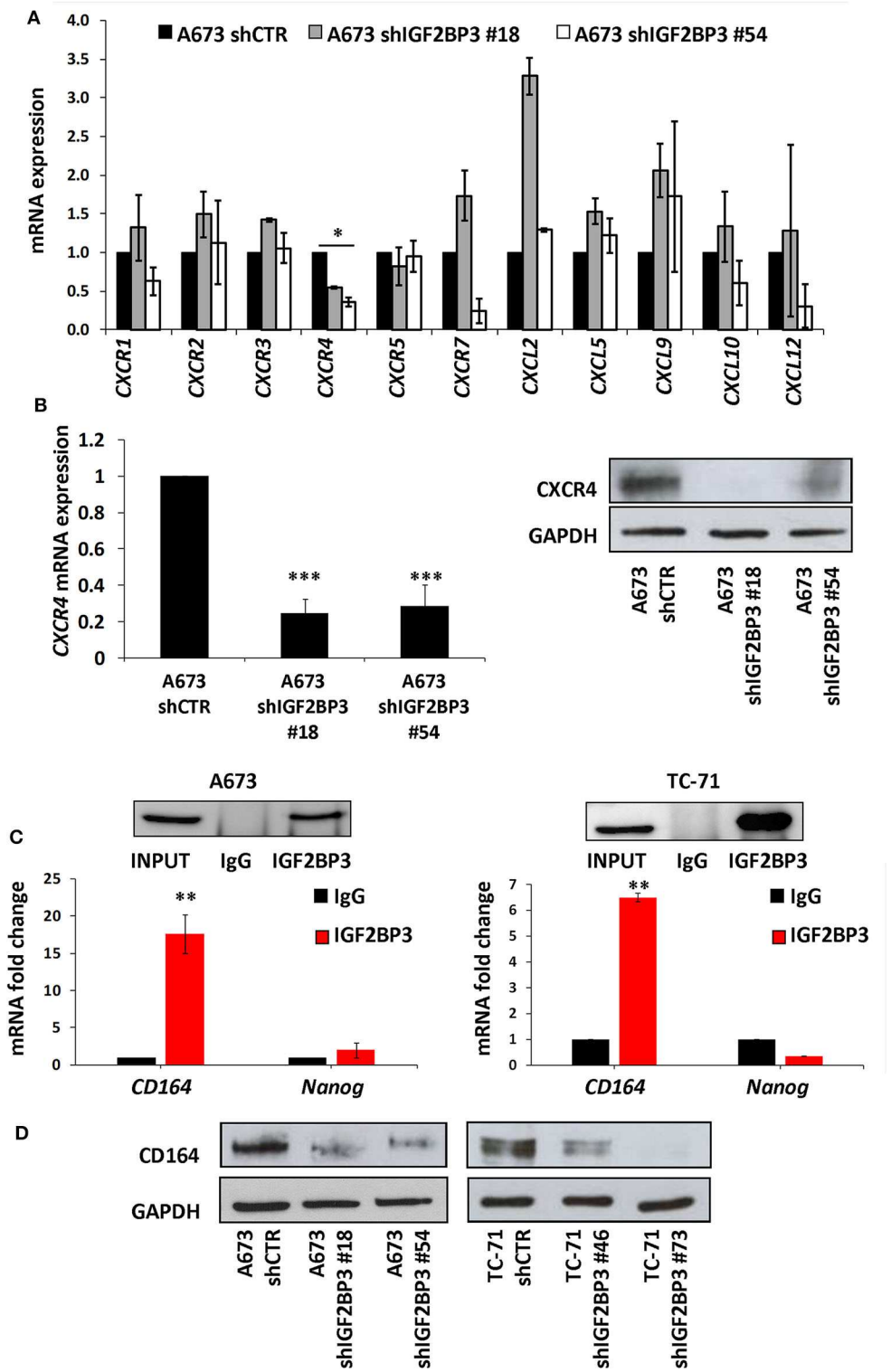

FIGURE 2 | Characterization of the IGF2BP3/CD164/CXCR4 oncogenic axis in EWS cells. **(A)** qRT-PCR analysis performed with RT^2 Profiler Cancer Inflammation and Immunity Crosstalk PCR Array on IGF2BP3-depleted or empty vector-transfected (shCTR) A673 EWS cells. Data are shown as $2^{-\Delta\Delta Ct}$ using A673 shCTR as calibrator and *GAPDH* as endogenous control. Mean ± *SE* of two independent experiments is shown. *$p < 0.05$, Student's *t*-test. **(B)** CXCR4 expression analyzed via (left) qRT-PCR or (right) western blot in IGF2BP3-depleted or empty vector-transfected (shCTR) A673 EWS cells. GAPDH was used as (left) housekeeping gene or (right) loading control. Histogram and western blot represent the sum of three independent experiments. ***$p < 0.001$, Student's *t*-test. **(C)** RIP assay performed on extracts from A673 and TC-71 EWS cells using an IGF2BP3 antibody or non-immune isotype matched IgG. *CD164* and *Nanog* mRNAs were quantified using

(Continued)

FIGURE 2 | qRT-PCR analysis. *Nanog* was used as a negative control. Western blot shows the specificity of IGF2BP3 antibody. Histograms represent mean ± *SE* of at least two independent experiments. $^{**}p < 0.01$, Student's *t*-test. **(D)** Western blot depicting CD164 expression on IGF2BP3-depleted or empty vector-transfected (shCTR) A673 and TC-71 EWS cells. Representative western blots are shown. GAPDH was used as loading control.

one-way ANOVA; **Supplementary Table 1**). This signature clearly separated the two groups with different *IGF2BP3* expression levels when hierarchical supervised clustering was performed (**Supplementary Figure 1**). Enrichment analysis using GeneGo annotation revealed the specific involvement of immunological and chemokine-mediated signaling pathways (**Table 2**).

IGF2BP3 Regulates the Expression of CXCR4 Through CD164

To confirm the functional association between IGF2BP3 and chemokine signaling pathways, we took advantage of IGF2BP3-depleted experimental EWS cell models previously generated by shRNA approaches (21) (**Supplementary Figure 2**). We profiled control-transfected and IGF2BP3-depleted A673 EWS cells for genes encoding chemokine receptors and ligands using the RT^2 Profiler Cancer Inflammation and Immunity Crosstalk PCR Array. Notably, only *CXCR4* was significantly downregulated in IGF2BP3-depleted A673 cells compared to controls (**Figure 2A**). Next, we confirmed by qRT-PCR and western blotting analyses downregulation of CXCR4 at mRNA and protein levels in IGF2BP3-silenced cells (**Figure 2B**), suggesting that CXCR4 might work as novel downstream effectors of IGF2BP3 action.

Data from the literature indicate that IGF2BP3 modulates the expression of CD164 (33, 34), a type 1 integral transmembrane sialomucin involved in the regulation of adhesion and migration of tumor cells (35, 36). Significantly, CD164 regulates CXCR4 function in different tumor types (36–38). Thus, we initially investigated a possible functional interaction between IGF2BP3 and *CD164* mRNA by RIP assay. In both A673 and TC-71 EWS cells *CD164* was significantly enriched in samples immunoprecipitated with anti-IGF2BP3 antibody as compared to IgG-immunoprecipitated control samples (**Figure 2C**). In addition, stable depletion of IGF2BP3 in A673 and TC-71 cells (**Supplementary Figure 2**) was associated with a significant reduction of CD164 protein expression levels as demonstrated by immunoblot analysis (**Figure 2D**). Next, we analyzed by qRT-PCR *IGF2BP3*, *CD164*, and *CXCR4* expression levels in clinical samples. We confirmed statistical association among the three molecules in both the 48 primary localized tumors and 44 metastatic lesions previously described (**Figures 3A–F**). Because Spearman coefficients (*r*) still indicated a weak to moderate correlation between *IGF2BP3* and *CD164* or *CXCR4* while a strong correlation between *CD164* and *CXCR4* (32), we further investigated the IGF2BP3/CD164/CXCR4 association by IHC in an independent cohort of 50 primary tumors (**Table 1**). The analyses confirmed a significant association at protein level between CD164 expression with both IGF2BP3 ($p = 0.05$, Chi-square test) and CXCR4 ($p = 0.04$, Chi-square test) (**Table 3**, **Supplementary Figure 3**).

Taken together these data support a role of IGF2BP3 in regulating the CD164/CXCR4 complex and demonstrate the evidence of an IGF3BP3-CD164-CXCR4 oncogenic axis critical for EWS progression.

The IGF2BP3/CD164/CXCR4 Axis Affects Migration of EWS Cells in Response to CXCL12 and Under Hypoxia Conditions

While the role of CXCR4 in regulating migration of EWS cells has been previously established (13, 15), there are no data at the moment supporting the role of CD164 in modulating EWS cancer cells motility. Thus, we used siRNA approaches and transiently depleted CD164 in A673 and TC-71 cells. We obtained a robust CD164 depletion in both cell lines (**Figure 4A**), which determined a significant inhibition of EWS cell motility in condition of chemotactic stimulus toward a CXCL12 gradient (**Figure 4B**), supporting the notion that CD164 might act as an adjuvant factor of CXCR4 signaling in EWS cells.

We then investigated by confocal microscopy whether CD164 and CXCR4 might colocalize in A673 cell line. In CXCL12-unstimulated cells, a homogeneous distribution of CD164 and CXCR4 was observed in the cytoplasm and at the plasma membrane (**Figures 5A,B**). On the contrary, upon CXCL12 stimulation, CD164 and CXCR4 colocalized at the plasma membrane. To confirm that CD164 and CXCR4 indeed interacts at the plasma membrane, we repeated colocalization experiments supplementing CXCL12 with the general endocytosis inhibitor dynasore, a GTPase inhibitor that blocks dynamin activity, thus affecting both clathrin-dependent and -independent endocytic pathways (39). The combination of CXCL12 and dynasore enhanced colocalization of CD164 and CXCR4 (**Figures 5A,B**), confirming that this interaction likely occurs at the plasma membrane of A673 cells (**Figure 5A**, white arrows). Collectively these results suggest that CD164 and CXCR4 colocalize at the plasma membrane of A673 cells in CXCL12-dependent fashion.

Because CXCR4 is induced in EWS cells exposed to hypoxia (13), a common condition of human tumor microenvironment (40), we investigated the contribution of the IGF2BP3/CD164/CXCR4 axis on CXCL12-evoked biological responses of EWS cells under normoxic (21% O_2) or hypoxic conditions (1% O_2). In line with previous evidence (13), EWS cells exposed to hypoxia showed induced expression of CXCR4 and of the hypoxia inducible factor alpha (HIF-1α), used as control (**Figure 6A**). Interestingly, IGF2BP3-silenced cells did not show CXCR4 expression, which was not increased under hypoxic conditions (**Figure 6A**). From the functional

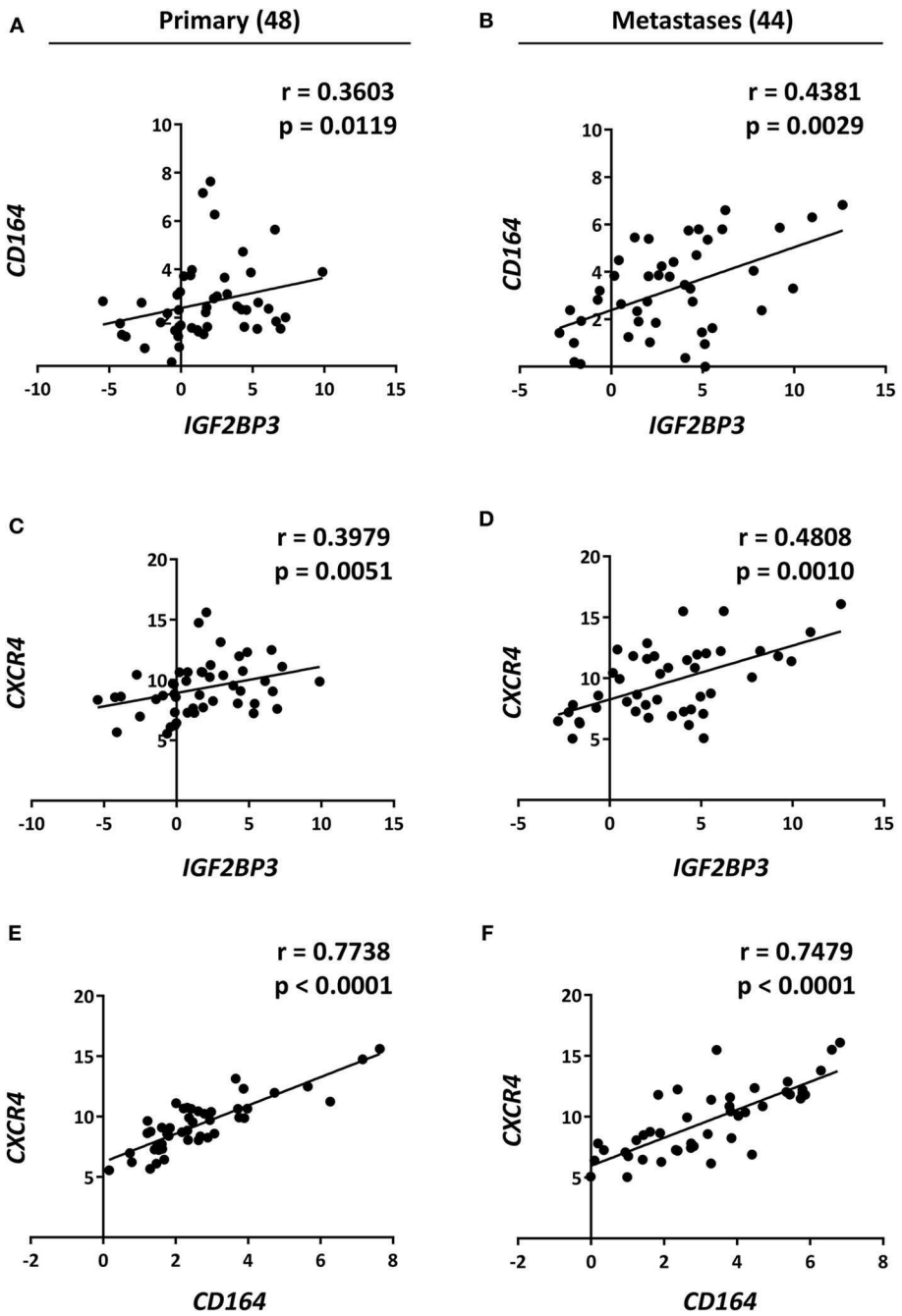

FIGURE 3 | Analysis and correlation of *IGF2BP3*, *CD164*, and *CXCR4* mRNA levels in EWS patients. Scatter plots displaying correlations between *IGF2BP3*, *CD164*, and *CXCR4* mRNA levels in EWS **(A,C,E)** primary tumors and **(B,D,F)** metastatic lesions analyzed via qRT-PCR. Number of analyzed cases is reported above each column. Relative mRNA expression reported as $\log_2$ is shown. Human mesenchymal stem cells were used as calibrator. Correlation coefficient (*r*) and *p*-value were calculated using Spearman's rank test.

standpoint, the inhibitory effect on cell migration associated with IGF2BP3 depletion was amplified under hypoxic conditions. In fact, A673 cells silenced for IGF2BP3 showed reduced migration in response to CXCL12 either in normoxic or under hypoxia conditions (**Figure 6B**). Of note, the reduction was more evident in hypoxia condition ($p = 0.005$, one-way ANOVA), indicating that the impact of reduced expression of the IGF2BP3/CD164/CXCR4 oncogenic pathway may be stronger in the tumor microenvironment compared to physiological conditions.

DISCUSSION

EWS is characterized by a very low somatic mutational load (6–8) and high levels of inter- and intratumor epigenetic heterogeneity (10–12). Analysis of DNA methylation has highlighted a large spectrum of alterations, which reflect disease heterogeneity in term of stem cell differentiation and clinical outcome, and preclude the possibility of identifying subset of patients with differential risk of progression (12). Treatment of EWS is still based on high dense chemotherapy, with relevant impact on quality of life of survivors, who may be overtreated, and on outcome of high-risk patients, who should be considered for alternative drug regimens.

We have recently demonstrated that the mRNA binding protein IGF2BP3 constitutes an important biomarker for EWS (20, 21) as in fact high expression of IGF2BP3 in primary tumors is associated with poor prognosis of EWS patients (21). In addition, we demonstrated that IGF2BP3 increases anchorage-independent growth and migration of EWS cells (21) suggesting a putative role for IGF2BP3 as molecular driver of EWS progression. In this study, we demonstrated that: (A) High *IGF2BP3* mRNA expression levels correlate with EWS metastasis. (B) EWS tumors with high *IGF2BP3* mRNA expression levels are characterized by a specific gene signature enriched in chemokine-mediated signaling pathways. (C) IGF2BP3 regulates the expression of CXCR4 through CD164. (D) CD164 and CXCR4 colocalize at the plasma membrane of EWS cells upon CXCL12 stimulation. (E) IGF2BP3, CD164, and CXCR4 expression levels correlate in clinical samples. (F) The IGF2BP3/CD164/CXCR4 oncogenic axis promotes motility of EWS cells in response to CXCL12 and under hypoxia conditions.

Previously published data from our laboratory indicates that IGF2BP3 may exert its oncogenic action in EWS in both IGFs-dependent and -independent manner. IGF2BP3 loss promoted IGF1R downregulation and inhibited IGF1-evoked biological responses, thereby reducing cell growth and motility of EWS cells (20). IGF1R loss was associated with a compensatory mechanism driven by activation of the insulin receptor isoform A (IR-A) and its cognate ligand IGF2, which conferred enhanced sensitivity to dual IGF1R/IR inhibitors (20). On the other hand, IGF2BP3 expression is predictive of poor prognosis of EWS and regulate EWS aggressiveness independently of IGF1R action (21). The data presented here support the novel observation that in EWS cells IGF2BP3 might be a critical factor in regulating a specific cytokine pathway consisting of CD164 and CXCR4 signaling.

A role for CXCR4 in EWS has been previously demonstrated (13, 15, 41). Expression of CXCR4 is highly dynamic in EWS, and can be transiently induced by exposure to microenvironmental stress, like starvation, growth constraint and hypoxia (13). EWS cells characterized by high CXCR4 expression levels show increased invasion and migration capability, partially mediated by the intracellular activation of the Rho-GTPases, Rac1, and Cdc42 (13). Significantly, targeting the CXCL12/CXCR4 axis inhibited the aggressive phenotype, thereby indicating a potential contribution of CXCR4 signaling to EWS metastasis (13). In addition, in the model presented by Krook et al. stress induces the conversion of CXCR4-negative EWS cells to CXCR4-positive cells, thereby supporting the role of the CXCL12/CXCR4 signaling pathway in tumor progression (15). This switch is mediated, at least in part, by epigenetic modifications of the *CXCR4* promoter, which transitions from an inactive bivalent state to a univalent active state (15).

The adhesion receptor CD164 (endolyn), belonging to the sialomucin family, regulates the adhesion of $CD34^+$ cells to bone marrow stroma, and the recruitment of those cells into cycle (37). CD164 associates with CXCR4 and cooperates with it in promoting CXCL12-mediated cell migration (37). CD164 depletion significantly attenuated the PI3K pathway but it did not alter MAPK activation, suggesting pathway

TABLE 3 | Association between CD164, CXCR4, and IGF2BP3 according to Chi-square test in 50 primary localized EWS cases analyzed by IHC.

CD164	Negative	Positive	*p*-value
CXCR4			0.04
Negative	5	7	
Positive	5	30	
IGF2BP3			0.05
Negative	6	7	
Positive	7	30	

CXCR4, not evaluable in three cases.

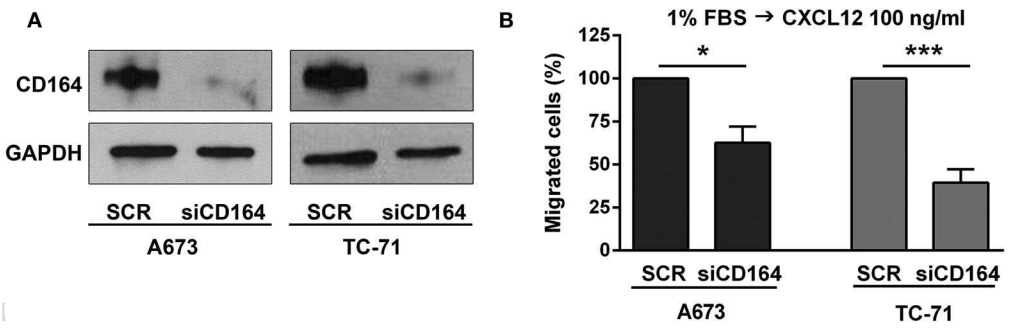

FIGURE 4 | Functional relevance of CD164 in EWS cells. **(A)** CD164 silencing was achieved in A673 and TC-71 EWS cells after 72 h of transfection of siCD164 (80 nM) or scrambled control siRNA (SCR; 80 nM). GAPDH was used as the loading control. **(B)** Histogram shows the migration of A673 and TC-71 cells treated with siRNA or SCR using a CXCL12 (100 ng/ml) gradient. Mean ± *SE* of at least two independent experiments is shown. $*p < 0.05$; $***p < 0.001$, Student's *t*-test.

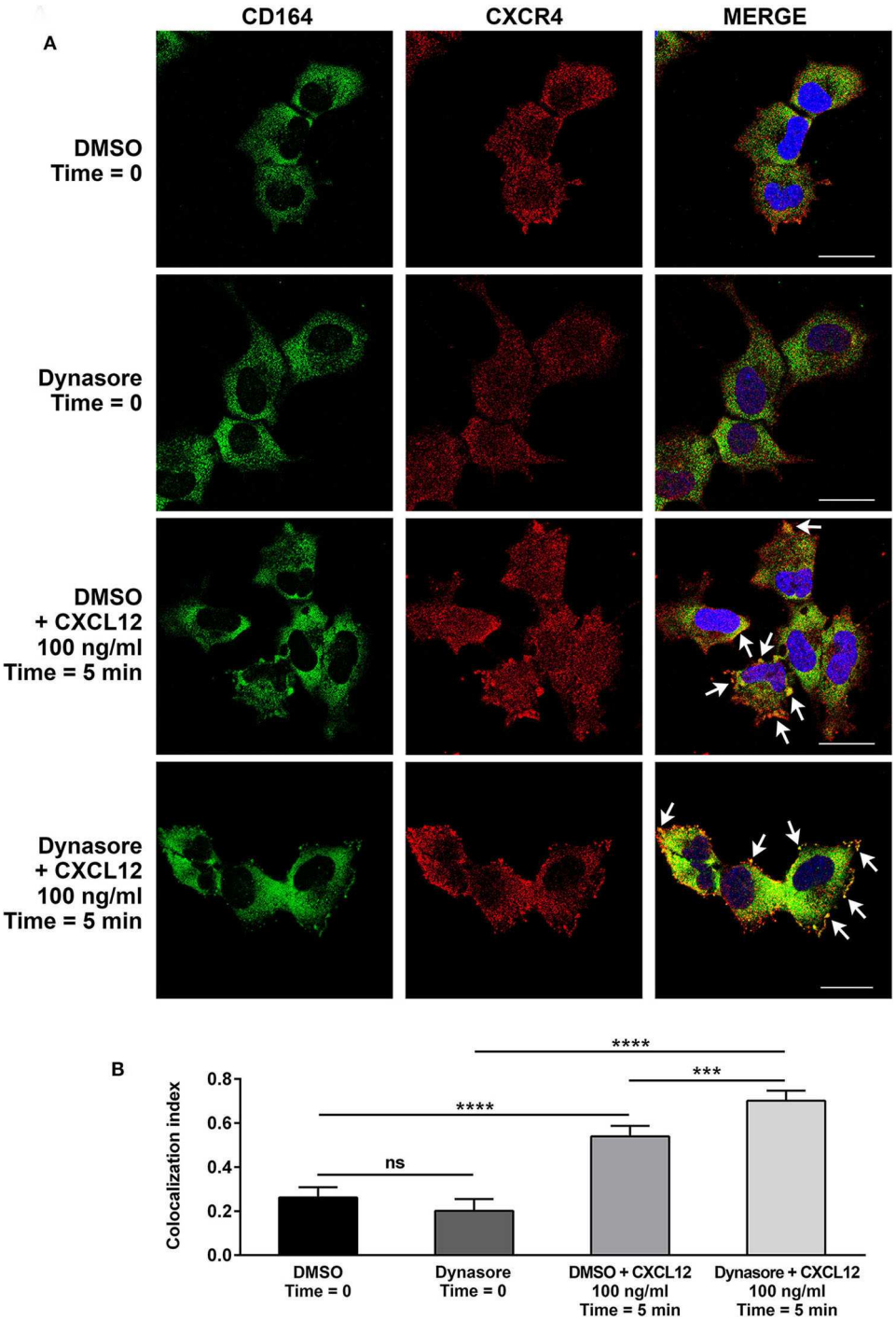

FIGURE 5 | CD164 and CXCR4 colocalize upon CXCL12 stimulation in EWS cells. **(A)** Colocalization of CD164 and CXCR4 was assessed in A673 cells by immunostaining and confocal microscopy. Cells were pretreated with dynasore (80 μM), or DMSO as control, and then stimulated with CXCL12 100 ng/ml for 5 min (Time = 5 min), or left unstimulated (Time = 0). Images were taken using confocal microscopy and representative single *Z*-stack pictures are shown (scale bar 25 μm). **(B)** *Z*-stacks were analyzed for colocalization by NIS Elements AR4.20.01 software (Nikon). Colocalization index is represented by histograms. Mean ± *SE* of an average of 30 independent fields is shown. ns, not significant; ***$p < 0.01$; ****$p < 0.0001$, one-way ANOVA.

specificity of CD164 action (37). A tumorigenic role of CD164 has been demonstrated in ovarian cancer where CD164 is upregulated in malignant ovarian cancer cell lines (38). CD164 overexpression in human ovarian epithelial surface cells increased CXCL12/CXCR4 expression, enhanced cellular proliferation, and colony formation, and suppressed apoptosis

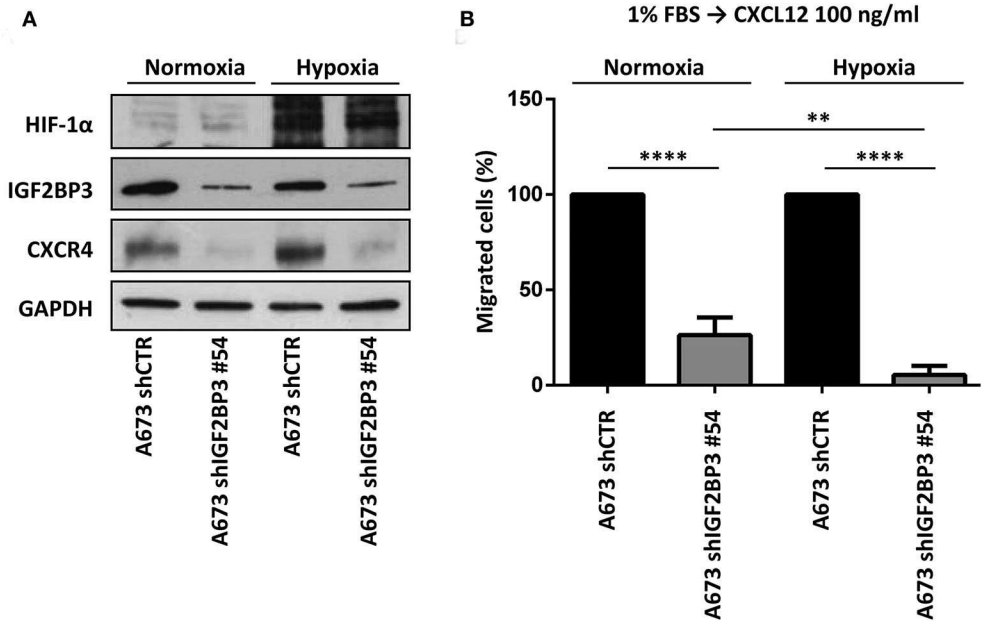

FIGURE 6 | Functional relevance of IGF2BP3/CXCR4 axis in EWS cells. **(A)** Western blotting displaying HIF-1α, IGF2BP3, and CXCR4 expression in IGF2BP3-depleted or empty vector-transfected (shCTR) A673 EWS cells grown for 72 h under normoxia (21% O_2) or hypoxia (1% O_2). The western blots represent the sum of three independent experiments. GAPDH was used as the loading control. **(B)** Migration of IGF2BP3-depleted or empty vector-transfected (shCTR) A673 EWS cells using a CXCL12 (100 ng/ml) gradient under normoxia (21% O2) or hypoxia (1% O2). Mean ± *SE* of three independent experiments is shown. $^{**}p < 0.01$; $^{****}p < 0.0001$, one-way ANOVA.

(38). Clinicopathological correlation analysis additionally indicated that CD164 upregulation was significantly associated with tumor grade and metastasis. In EWS, a putative role for CD164 in EWS transformation was suggested by Grunewald et al. who demonstrated that the thyroid receptor interacting protein 6 (TRIP6), belonging to the Zyxin family of proteins, is overexpressed in EWS and promotes cell growth, invasion, and migration through a transcriptional pro-invasive gene signature, which included *CD164* (42). However, CD164 mechanisms of action in EWS cells were not further characterized and its impact on tumor progression has never been evaluated.

According to previous evidences (33, 34), our data confirm a direct functional interaction between IGF2BP3 and CD164. In fact, IGF2BP3 and *CD164* are part of a complex detected by RIP assays, suggesting that IGF2BP3 might regulate mRNA stability and therefore expression levels of CD164. In turn, CD164 functionally interacts with CXCR4, thus regulating CXCR4 activation and CXCL12-dependent motility of EWS cells. In ovarian cancer cells, CD164 was localized in the cytosol and nucleus suggesting that nuclear CD164 might regulate CXCR4 promoter activity (38). The definition of downstream mechanisms of action of this signaling axis in EWS cells deserves further studies. It is important to mention that, in addition to IGF2BP3, additional proteins may contribute to CD164/CXCR4 regulation at post-transcriptional or epigenetic level, as suggested by the moderate associations between these 3 molecules observed in EWS cases. For instance, CXCR4 is regulated by dynamic post-translational histone modifications (15) while CD164 is a direct target of miRNA124, whose role in EWS has been previously reported (43, 44). Here, we put emphasis on the definition of an axis that may favor metastasis formation, the critical medical issue in the cure of EWS patients, and we provide evidence that support the possible use of drugs targeting IGF2BP3 and/or CXCR4 in high-risk patients with high expression of IGF2BP3/CD164/CXCR4 molecules. As recently reported, CXCL12 favors a pro-metastatic bone marrow niche in multiple myeloma, as well as in solid tumors with propensity to give bone metastases, including gastric, medullary thyroid, lung, prostate, and renal carcinomas (45). CXCR4-blocking agents, such as the neutralizing antibody MDX1338 or Ulocuplumab, were reported to efficiently reduce migration and invasion of osteosarcoma, alveolar rhabdomyosarcoma and myeloma cells and suppress the CXCR4-driven Epithelial-to-mesenchymal (EMT)-like phenotype (45–47), supporting the specific targeting of CXCR4 in therapy. More recently, the combination of MDX1338 and activated and expanded natural killer (NKAE) cell therapy was proposed as novel therapeutic approach to efficiently inhibit metastasis in mice (48). However, considering that CXCR4 may be up-regulated by epigenetic alterations or hypoxia-driven signaling which allow tumor cells to adapt and win the selection leading to tumor cell dissemination and metastasis in a new host environment, inhibition of IGF2BP3 may be more relevant. We have recently reported that inhibitors of Bromodomain and Extraterminal domain (BET) proteins can reduce expression of IGF2BP3 in EWS cells and synergize with vincristine (21). Further studies are necessary to develop more specific agents against this oncogenetic RBP.

In summary, the data presented in this work identified CD164 and CXCR4 as novel IGF2BP3 downstream functional

effectors supporting the notion that the IGF2BP3/CD164/CXCR4 oncogenic axis may work as critical modulator of EWS aggressiveness. In addition, IGF2BP3, CD164, and CXCR4 expression levels may work as novel biomarkers predictive of EWS progression. Targeting of this axis may effectively prevent EWS disease dissemination.

DATA AVAILABILITY STATEMENT

The RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (49) and are accessible through GEO Series accession number GSE150722. Microarray data are accessible through GEO Series accession number GSE12102 (9).

ETHICS STATEMENT

The studies involving human participants were approved by Ethical Committee of the IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy (0019012/2016, 0005175/2018, and 0006158/2020). Written informed consent to donate material to the IRCCS Istituto Ortopedico Rizzoli tissue biobank for research purposes was obtained.

AUTHOR CONTRIBUTIONS

CM and KS: conception and design of the study. CM, GC, MM, IR, and AP: acquisition of data. CM, GC, MM, AMM, AM, and KS: analysis and interpretation of data. CM, AM, and KS: drafting or revising the work. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00994/full#supplementary-material

Supplementary Table 1 | List of differentially expressed genes identified via RNAseq analyses in IGF2BP3-high vs IGF2BP3-low expressers primary localized EWS cases.

Supplementary Figure 1 | IGF2BP3 signature in EWS.

Supplementary Figure 2 | Evaluation of IGF2BP3 silencing in EWS cellular models.

Supplementary Figure 3 | IGF2BP3/CD164/CXCR4 association in EWS.

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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.

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METHODS
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Thymic Epithelial Tumors as a Model of Networking: Development of a Synergistic Strategy for Clinical and Translational Research Purposes

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Among the group of thymic epithelial tumors (TET), thymomas often show either uncertain or explicit malignant biological behavior, local invasiveness, and intrathoracic relapse and are often difficult to manage. From the initial stages, thymic carcinomas tend to show aggressive behavior and extrathoracic spread. Moreover, the interplay of epithelial cells and thymocytes in thymomas causes complex immune derangement and related systemic autoimmune diseases. Due to their rare occurrence and to the limited funding opportunities available for rare tumors, it is challenging to make advances in clinical and translational research in TET. The authors of this paper are all members of a multidisciplinary clinical and research thoracic tumor team. Strong input was given to the team by long-standing expertise in TET in the Pathology Department. In addition, thanks to the collaboration between research units at our Institute as well as to national collaborations, over the last 10 years we were able to perform several tissue-based research studies. The most recent studies focused on microRNA and on functional studies on the thymic carcinoma cell line 1889c. The recent implementation of our biobank now provides us with a new tool for networking collaborative research activities. Moreover, the participation in a worldwide community such as ITMIG (International Thymic Malignancy Interest Group) has allowed us to significantly contribute toward fundamental projects/research both in tissue-based studies (The Cancer Genome Atlas) and in clinical studies (TNM staging of TET). Our achievements derive from

constant commitment and long-standing experience in diagnosis and research in TET. New perspectives opened up due to the establishment of national [the Italian Collaborative Group for ThYmic MalignanciEs (TYME)] and European reference networks such as EURACAN, for an empowered joint clinical action in adult solid rare tumors. The challenge we face still lies in the advancement of clinical and basic science in thymic epithelial malignancies.

Keywords: thymic epithelial tumors, thymoma, thymic carcinoma, biobank, microRNA, TCGA, ITMIG, EURACAN

INTRODUCTION

Thymic epithelial tumors (TET) are a rare group of tumors, comprising thymoma (THY) and thymic carcinoma (TC), that have an incidence rate of 0.13/100,000 per population in the United States according to the National Cancer Institute's (NCI) Surveillance, Epidemiology, and End Results (SEER) program/SEER database (DB) (1). Population-based data were provided by the European cancer registries (CRs) participating in the RARECARE project: compared to that in the United States, TET showed a slightly higher incidence rate of 0.17/100,000 per population, and "malignant" thymomas accounted for 0.14/100,000. TC, a much rarer disease than THY, occurs with an incidence rate of 0.2–0.5 per million individuals (2). Data on the epidemiology of two families of rare thoracic neoplasias (epithelial tumors of thymus and mesothelioma of pleura and pericardium) for 27 European countries have been recently reported in more detail by the RARECARENet working group (www.rarecarenet.eu) Malignant TET showed (in the period 2000–2007) a 5-year survival of 64%, on average (3). Recent advances in tumor biology and pathology reveal that TET constitute a unique group of neoplasias deriving from the epithelial cell network of the thymus (TEC). The extraordinary properties and characteristics of this primary lymphatic organ have been firmly established in the last 60 years, after the discovery by Miller (4) and Good (5) of the unique thymic immunological functions. Due to its central role in the homeostasis of the immune system, it is not surprising that the tumors deriving from TEC are associated with derangement of the immune system (6). In 2015, the World Health Organization (WHO) changed the International Classification for Disease of Oncology (ICD-O) code associated with thymoma from the suffix /1 applied to the third edition classification (7) to the suffix /3 for the fourth edition (8). This change reflects our increased knowledge in the biology of TET and contributes to forming the statement that "all thymomas can behave in a clinically aggressive fashion" irrespective of tumor stage and should be considered malignant (9). In recent years, significant interest in TET has been shown all around the world, and much progress has been made in the last few years due to the activity of the International Thymic Malignancy Interest Group (ITMIG) scientific society (10, 11) (www.itmig.org) and to the International Association for the Study of Lung Cancer (IASLC). Due to the joint effort of ITMIG and of IASLC and to the contribution of several important DBs (12), TET for the first time were included in the TNM staging system (13). The new staging system relies on retrospective data from more than 10,000 TET cases observed all around the world (14). Specific interest raised toward these unique tumors was also due to the US NCI's inclusion of TET in The Cancer Genome Atlas (TCGA) project (15), only one of the few families of rare tumors considered. Moreover, due to the inclusion of TET in the rare cancers included in the G8 group (rare thoracic tumors) of EURACAN, the network of rare adult solid cancers in the European reference networks (ERNs) (http://euracan.ern-net.eu), significant progress in their management has to be expected over the next few years (16). Recently, we also joined the Italian Collaborative Group for ThYmic MalignanciEs (TYME) as a reference center for the diagnosis and treatment of TET (17).

We wish to point out that the driving force behind bringing new opportunities in rare tumor research and international collaborations to our local setting was the renewed commitment and long-standing expertise of the Pathology Department. Pathology now plays a major role in bridging the gap between tumor research and clinical management in every field of tumor research. This also applies to our Institute in relation to the TET family of rare tumors. We describe here our own developing workup within the clinical and scientific contexts of TET, focusing mainly on the surgical approach, on the pathological workup, and on the ongoing research activities in different fields. Recently, a renewed opportunity was offered by progressing from a "sample collection"-based biobank to an institutionally certified ISO9001:2015 biobank. We discuss here specificities, critical issues, and challenges, focusing on our surgical, pathological, and biobank activities, as these are the main players of translational research. We also briefly mention the research projects accomplished to date and discuss how we will implement and improve our model/strategy for making progress in the future.

MATERIALS AND EQUIPMENT

The surgical procedures cited for both open-access and mini-invasive approaches for TET were performed with the standard surgical instruments of a thoracic surgery operating room. Robotic thymectomy was performed by the da Vinci® surgical system (Intuitive Surgical Inc.).

Laboratory Methods and Equipment at the Pathology Department

Fixation of tumor specimens in 10% buffered formalin and routine laboratory techniques and equipment of a pathology

laboratory were adopted to fix and to process tumor samples. Hematoxylin–eosin (H&E) was the standard routine used for staining. The Aperio system AT2 (Aperio Leica Biosystems) (CE IVD) whole-slide scanner (400-slide capacity) was used to scan slides for digital pathology.

We mention here only the main equipment available at the Pathology Department; other platforms/equipment found in the collaborating laboratories are described in detail elsewhere (18, 19): Immunohistochemistry (IHC) at our Pathology Department is performed on BOND-III, the fully automated IHC platform (LEICA BIOSYSTEMS). Our molecular biological/genetic equipment includes (1) the Ion Gene Studio™ S5 series for next-generation sequencing (NGS, Thermo Fisher); (2) the Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher). The Platforms for MicroRNA study (Agilent 2,100 Bioanalyzer and "Affymetrix® Human Gene 2.0 ST Arrays 2.0," both from Affymetrix, Santa Clara, California) are of routine research use at our Oncogenomic and Epigenetic Research Unit.

Biobanking instruments include cryogenic systems, labeling machines, and barcode readers. Systems for cryopreservation include electric freezers (−80°C); liquid nitrogen storage systems; a dedicated biobanking software, EasyTrack2D®; and instruments used for quality control of biological samples in measuring various cellular components (DNA, RNA, and protein) (Bioanalyzer, Agilent Biotechnologies). All these sets of equipment are available within the dedicated spaces with controlled access. Our biobank is ISO certified (ISO9001:2015) (20).

METHODS

Care Pathway of TET

Between 2000 and 2019, 196 patients were recorded in our DB at the IRCCS Regina Elena National Cancer Institute (IRE), including demographic data, histologic type updated to 2015 WHO classification (8), surgical procedures, and the main outcome indicators. Cases evaluated for pathological diagnosis as a second opinion were recorded together with internal cases.

Diagnostic/Preoperative Workup

At our Institute, patients who have been identified with an anterior mediastinal mass all undergo physical examination and routine biochemical tests, an electrocardiogram (echocardiogram when indicated), chest X-ray, arterial blood gas analyses, and pulmonary function tests. A neurological protocol to exclude autoimmune diseases, particularly myasthenia gravis (MG) (21), is applied. After multi-slice computerized tomography (CT) scans (128 slices) are performed, the case is then discussed during multidisciplinary thoracic tumor board meetings together with a thoracic surgeon, pathologist, oncologist, anesthesiologist, radiotherapist, pneumologist, and chest radiologist. In case of indication to radical surgery, patients undergo cardiological, and pneumological evaluation of preoperative risk. Surgical indications are mainly based on patient clinical conditions and on the CT findings. Positron emission tomography (PET)–CT with fluorine-18 fluorodeoxyglucose (^{18}F-FDG), magnetic resonance imaging (MRI), and octreotide scan are not part of the routine preoperative workup but are additional exams (22). When a complete resection is possible, preoperative biopsy is not indicated (23, 24). In case of invasion of adjoining structures such as the anonymous vein, pericardium, superior vena cava, phrenic nerves, and pleural cavities, a diagnostic biopsy is required; after the diagnosis by surgical biopsy or by fine needle biopsy aspiration (FNAB), the patient is usually referred to induction chemotherapy (25, 26) or to surgical treatment in combination with radiotherapy.

Surgical Approach

Sternotomy and, in selected cases, thoracotomy represent the first surgical options because they allow an open extended resection of mediastinal masses and surrounding tissues, including mediastinal fat around the great vessels (27). However, in the last two decades, minimally invasive techniques took progressive place into clinical practice by a growing number of surgeons (28, 29). Minimally invasive techniques include video-assisted thoracoscopic surgery (VATS) (30) and robotic-assisted thoracoscopic surgery (RATS) (31). According to TYME, minimally invasive surgery is recommended for a tumor dimension smaller than 5 cm (17); however, also in case of invasion of neighboring organs (the pericardium, lungs, mediastinal pleura, or phrenic nerve), this procedure is not a contraindication in expert hands (23). The objective is quite similar for both RATS and VATS approaches: to perform standard extended thymectomy, including the thymus and the surrounding mediastinal fatty tissue, en bloc.

Biobank: A Bridge Among Clinical and Scientific Resources

The process of biobanking starts once a patient suspected of having mediastinal masses for thymic malignancy is identified and gives his/her institutional review board (IRB)-approved informed consent to preserve samples in our biobank. The consent is signed by both the patient and surgeon. A request for banking biological fluids is prepared prior to the surgical intervention by the surgeon through the creation of a computerized order entry to the Biological Fluids Biobank in the Clinical Pathology Laboratory. Blood samples (whole blood, serum, and plasma) are withdrawn by research nurses in the surgical ward (prior to operation and during follow-up to outpatients). The sterile tissue specimen is immediately collected from the operating room and taken to the Tissue Biobank in the Pathology Department upon removal. After checking and testing for biomaterial conformity and adequacy for diagnosis, sampling is performed by a "dedicated" pathologist (32). Each specimen is sampled depending on size and quality of the tissue; consecutive samples are prepared. The selected samples are immediately snap-frozen in liquid nitrogen or are frozen in optimal cutting temperature (OCT) and stored at −80°C. The procedure applies to both resected surgical specimens and biopsies (when sufficient material is available). Representative corresponding samples like morphological controls from either the tumor or the peritumoral thymus—when available—are fixed in formalin overnight (at 4°C) (minimum 24 h) and embedded in paraffin [formalin-fixed, paraffin-embedded (FFPE) material]

(33) in a specific biobank archive. Tissue specimens are processed and stored in our tissue biobank by our "biobankers" according to the biobank standard operating procedures (SOPs) compliant with ISO9001/2015 certification (20). For sample collection and storage, clinical and biological data are recorded and managed by a dedicated software EasyTrack2D® according to the specific biobank SOPs. The quality of different fractions/samples (snap-frozen/OCT frozen/FFPE) is periodically evaluated for the preservation and yield of the cellular components by checking RNA/DNA extracted with RNA integrity number (RIN) (34). **Figure 1** shows the RIN value of some of our sample RNAs. Recently, our biobank group has introduced the collection and isolation of tumor cells from fresh tumor specimens/neoplastic effusions (35). As for TET, we are setting up primary tumor cell cultures (preliminary data, not shown).

Pathology—Diagnostic Workup and Digital Imaging

The recommendations of C.A. Moran and S. Suster (36) and of the International Collaboration on Cancer Reporting (ICCR) (37) in tumor sampling are followed (one tissue sample per centimeter of tumor or a minimum of 10 blocks for very large tumors). The peritumoral thymus is investigated by a "dedicated" pathologist who accurately and thoroughly examines the specimen and performs multiple sample embedding of peritumoral thymic fat tissue. In regard to pathological reporting, the 2015 WHO classification (8, 38) together with the ICCR recommendations (37) are followed. IHC plays a role in the diagnostic workup for diagnosis of thymomas with ambiguous histology and for the distinction between thymomas and thymic carcinomas (38). Pathological staging is performed by the pathologist on the basis of the tumor extent according to the eighth TNM (14, 39–41) published in its final and official version in 2017 (13). The Pathology Department, equipped with the Leica digital pathology platform Aperio AT2 (Aperio Leica Biosystems), performs most routine scans of representative slides of TET cases. Each H&E or significant IHC slide is scanned at a magnification of × 40. The scanning parameter settings are the default instrument settings. Digital images are analyzed by using the ImageScope® software. The image management system is the eSlide Manager® (12.3.3.5049) (Aperio Leica Biosystems).

Research Pathway in TET

For molecular pathology, the methods applied in our tissue-based studies are only briefly mentioned here; the reader is referred to the original publications (18, 19, 42–44). We used sequencing and *egfr* fluorescence *in situ* hybridization (FISH) to genotype our series of thymomas: (I) for polymorphisms and somatic loss of heterozygosity of the non-coding *egfr* CA-SSR-1 microsatellite and (II) for *egfr* gene copy number changes. More recently, for our NGS study, we used the Ion AmpliSeq Cancer Hotspot Panel v2 targets 50, which is the most commonly used cancer panel adopted for solid tumors in order to identify mutations indicating sensitivity and resistance to targeted therapies. The panel is able to identify more than 2,800 COSMIC hot spots of 50 genes, as described in several studies (45, 46). For the microRNA study, microRNA expression profiling of FFPE tumor tissue and peritumoral thymus was performed by microarray analysis; mRNA expression profiling of fresh frozen TET and peritumoral thymus was performed by microarray analysis. The role of miR-145-5p in TETs was evaluated *in vitro*, modulating its expression in a thymic carcinoma (1889c) cell line. The epigenetic transcriptional regulation of miR-145-5p was examined by treating the cell line with the HDAC inhibitor valproic acid (VPA) (19).

RESULTS

Between 2001 and 2019, 196 cases of TET were recorded, excluding non-neoplastic thymic disease cases, in adult patients. The data reported in **Table 1A** exclude lymphoid neoplasias occurring or involving the thymus, such as Hodgkin lymphomas as well as non-Hodgkin lymphomas and the relatively common metastatic disease to the thymus/anterior mediastinum. Primary non-epithelial as well as non-lymphoid tumors were rarely diagnosed in the thymus (47). **Table 1A** briefly reports basic demographical data and subtype distribution of 188 TET cases seen at our institution. A slight increase in cases per year was recorded from 2016 (**Table 1B**). Most of the TET cases were surgically treated at our Institute. Cases involving second opinions were also included. Most of them derived from regions of Central or Southern Italy and were shared for second opinion diagnostic purposes from the Rare Cancer Center of the Regione Campania (CRTR). However, recently, cases referred to the NCI in Milan (INT), within the TYME network, were also shared with us and examined for a second opinion. Our Institute is a participating reference center both for diagnostic activity on TET in Italy within the TYME network (17) and for the pathological assessment of cases within a biological translational study (BIOTET) designed by the NCI in Milan (48).

Most cases, including those referred for a second diagnostic opinion and treated at IRE, are evaluated and discussed at the multidisciplinary thoracic tumor board (49). At our Institute, we apply consolidated surgical procedures, thymectomy being the cornerstone surgical approach used for treating patients with TET. According to international guidelines, the open approach is the first choice (23, 27); however, VATS and RATS (**Table 2**) also play a relevant role in our approach to thymic surgery. In our clinical practice, we routinely perform the RATS left approach for left-sided and central mediastinal lesions and reserve the right approach for right-sided tumors. The main advantages of this type of technique include the three-port access through 1-cm incisions, CO_2 inflation in the mediastinum that radically increases operating space, accuracy of instrument movement under mechanical control, and 2D stereoscopic full-HD vision. Moreover, in the last few years, we have moved on from using the three-port VATS to the uniportal VATS. In comparison to RATS, the uniportal VATS approach, used only for small lesions with no invasion to adjacent structures (50, 51), even though slightly less accurate, has direct control over surgical instruments, returning to the tactile feedback of the surgeon's hand. Moreover, the uniportal access technique shows relevant post-operative

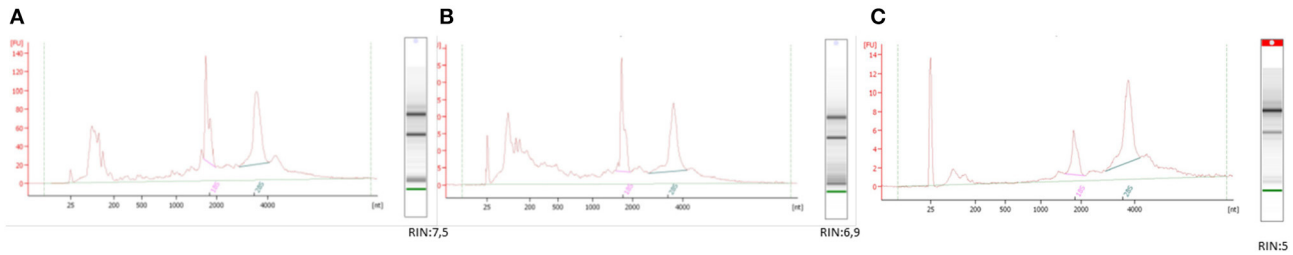

FIGURE 1 | Methods in biobank: ID 528090BIOSPECIMEN QUALITY CONTROL. The image shows the quality of the different TET samples after RNA extraction using the different processing protocols **(A)** snap-frozen, **(B)** OCT, and **(C)** FFPE. The panel shows representative electropherograms of each sample type. The RIN values from each category were different between the groups (protocols A and B produced RIN values of ≥5, protocol C produced RIN values of ≤5—only moderately degraded RNA). All three methods guarantee the integrity of the RNA, rendering it suitable for most types of downstream applications.

TABLE 1A | Distribution by sex and histotypes of TET cases according to the 2015 WHO classification in the period 2001–2019—TET PATIENTS tot 196; TET, not further classifiable: 8 cases; Male: 100 (51%); Female:96 (49%).

WHO histologic type (*n* = 188)	
A	17 (9%)
AB	47 (25%)
B1	13 (7%)
B2	61 (33%)
B3	12 (6%)
Thymic carcinoma	38 (20%)

TABLE 1B | TET case number/year in the last years.

Year	**TET Total case no.**
2014	14
2015	11
2016	24
2017	26
2018	17
2019	18

TABLE 2 | Distribution/year of thymectomies by RATS at IRE in the period 2016–2019.

	2016	**2017**	**2018**	**2019**
Robotic thymectomy	9	12	13	12

pain reduction and better aesthetic results in comparison to the open approach.

Pathological TET Evaluation and Research Activities

For tumor diagnosis, classification, and digital imaging, in all cases, surgical specimens as well as bioptic material are classified according to the 2015 WHO classification, and the B2 subtype was the most represented histotype (33% of recorded cases) (**Table 1A**). Tumor tissue is routinely extensively sampled, and even though the amount of lymphocytes and/or thymocytes might vary in different areas of THY, the histological variation does not affect the main TET subtyping, performed according to the criteria set out in the 2015 WHO classification (8, 38). Moreover, extensive sampling allows the availability of FFPE material not only from the tumor itself but also from the peritumoral thymus, whenever remnant tissue is available. We provide blocks with "key-blocks" in order to evaluate the tumor and its surrounding tissue for accurate staging (37). Anterior mediastinal lymph nodes are also included in the sampling, because they are usually removed by the surgeons together with the fat tissue of the anterior mediastinum (52). In surgically treated THY cases at IRE, we found a metastasis in only one case, in a laterocervical lymph node (53), which developed 9 years from the original diagnosis. Recently, the use of the digital pathology is growing at an exponential rate, and we have been scanning most of the representative slides.

Tissue-based research activity in TET at IRE was first based on a tissue microarray (TMA)-based immunohistochemical study of vascular endothelial growth factor receptors (VEGFR family) in 200 cases from different Italian institutions. The TMA study provided evidence that tissue receptors of the VEGFR family are distributed among TET subtypes, reaching the maximum expression in TC (18). Subsequently, in a pilot study carried out on the *egfr* microsatellite CA-SSR-1 performed by the first genetic analyzer available in pathology, Thermo Fisher's 3130 genetic analyzer, we were able to show that CA-SSR-1 allelic imbalance with short allele relative prevalence significantly correlated with EGFR 3+ immunohistochemical scores, increased *egfr* gene copy numbers, and advanced stage with relapsing/metastatic behavior in thymomas (44). More recently, we have established further collaborations with other in-house research units (43) and national (19, 42, 54) and international institutes (55, 56). Thanks to frequent participation in meetings and interfacing with members of the scientific community at major conferences on thymic tumors, as well as holding structured workgroups supported by the scientific society ITMIG, our boundaries have changed and widened. The TCGA-THYM study participation is an example of a major cornerstone. This study, among other results, demonstrated the existence of four molecular subtypes in TET, which corresponded to the morphological subtypes in the WHO classification (57). In-house, we started an NGS

TABLE 3 | List of samples collected from 2017 to 2019 in our Biobank deriving from thoracic tumors.

Department	Pathology	Patients	Sample preservation mode					
			Tumor tissue cryopreservation	Peritumoral tissue cryopreservation	Tumor tissue OCT	Peritumoral tissue OCT	FFPE	Total
Thoracic surgery	Thymoma	22	169	49	6	4	20	248
	Lung tumors	152	801	711	65	39	130	1,746
	Mesothelioma	2	8	0	1	0	1	10
	Lymphoma	8	38	4	1	0	8	51
	Pleural effusion	35	0	0	0	0	0	0
	Peripheral blood (pleural effusion)	22	0	0	0	0	0	0
Total		241	1,016	764	73	43	20	2,055

In addition to other thoracic tumors, specifically, 248 TET tissue samples from 22 patients were collected, of which 169 samples of snap frozen tumor tissue, 49 samples of adjacent normal snap frozen tissue. Moreover, we collected 6 samples of tumor tissues preserved in Optimal cutting temperature (OCT) and 4 samples of adjacent "peritumoral thymus" stored in OCT.

study in order to map the genomic alterations of our TC series; preliminary data were presented at the most important conferences held on TET or at IASLC WCLC (58, 59).

For biobanking and TET frozen tissue-based research (60), the Thoracic Surgery Unit and the Pathology Department between September 2017 and May 2019 provided our biobank with tumor tissues from over 241 patients with thoracic tumors, including the most common lung carcinoma; TET; mesothelioma; and thoracic lymphoma (**Table 3**). At present (02/2020), we have 263 stored tumor samples from 31 patients affected with TET. The tumor samples preserved as morphological control and fixed in formalin at 4°C provided better morphological results than routine specimens (**Figures 2**, **3**) (61). In the same period, at the Biological Fluid Biobank, we started to collect peripheral blood (PB) and serum/plasma from TET patients, thus preserving in the biobank complete samples (tumors and germline tissue) from 26 TET patients. Moreover, in the last few years, also before establishing our institutional biobank, we provided high-quality material from our "frozen collection of cases" to a gene expression profile carried out in our national scientific collaboration on microRNA. By analysis of a series of TET samples and peritumoral thymus, we identified a 69-gene signature of miR-145-5p putative target mRNAs. These mRNAs are differentially expressed between tumor and peritumoral thymus, and their expression is inversely correlated to that of miR-145-5p. Moreover, we evidenced that the epigenetic treatment of TC cell line 1889c with VPA, a histone acetylation inhibitor, resulted in the induction of miR-145-5p expression and downregulation of its target genes, showing antitumor effects in TET (cell cycle arrest and reduction of cell viability, colony-forming ability, and migration capability) (19).

DISCUSSION

The thymus is a primary lymphatic organ which sees the beginning of thymic involution at puberty (62), yet seeding, in an adult age, epithelial tumors deriving from highly specialized cells (63) of fundamental biological and pathological relevance. Our Institute has a long-standing interest in the diagnosis of thymic and mediastinal lesions (8, 38, 47) and management of TET patients (26, 42). Moreover, our Institute is well-known as an Italian expert center for the surgical and multimodality approach applied for the removal of mediastinal masses (25). At present, we play an active leading role in TYME, the main Italian network for thymic tumor management (17, 64), and we will be contributing to the ongoing ninth TNM staging project of thymic tumors and lung carcinoma, expected in 2024 (65, 66). In EURACAN, the G8 network, we contribute to ongoing activities in the clinical patient management system (CPMS), a web-based complex clinical software, and to the Digital Pathology Task force, and research projects are moving forward (16); currently, EURACAN in conjunction with the European Organization for Research on Cancer (EORTC) are moving ahead. EORTC, through SPECTA, an academic translational research infrastructure for biomaterial collection, aims to promote a comprehensive molecular profiling and virtual central pathology review also in the field of rare thoracic tumors.

Translational Research Perspective

In our experienced clinical setting, over the past few years, we have applied multiple approaches toward TET tissue-based research studies. The TET biological system requires particular attention due to the occurrence of strictly intermingled epithelial and lymphoid cells in tumors. Therefore, IHC shows advantages because cells labeled with biomarkers are singularly identified. In our multicenter study on a series of 200 TET cases collected in the larger TET-TMA series built up, an extensive immunohistochemical angiogenesis-related investigation showed that VEGFR expression was associated with invasiveness and advanced stage (18). These data could provide biological support for the use of anti-angiogenetic drugs in TET treatment (67, 68). An Italian clinical trial exploring the role of angiogenetic receptors in TET is currently in progress (48).

Molecular and genomic studies, on the other hand, require attention in using TEC-enriched samples. In our pilot study focusing on the *egfr* relevance in the pathogenesis of TET, we provided statistically significant insight on the possible role that the length of the *egfr* microsatellite CA-SSR-1 and the *egfr* gene copy number could play in TET growth (44).

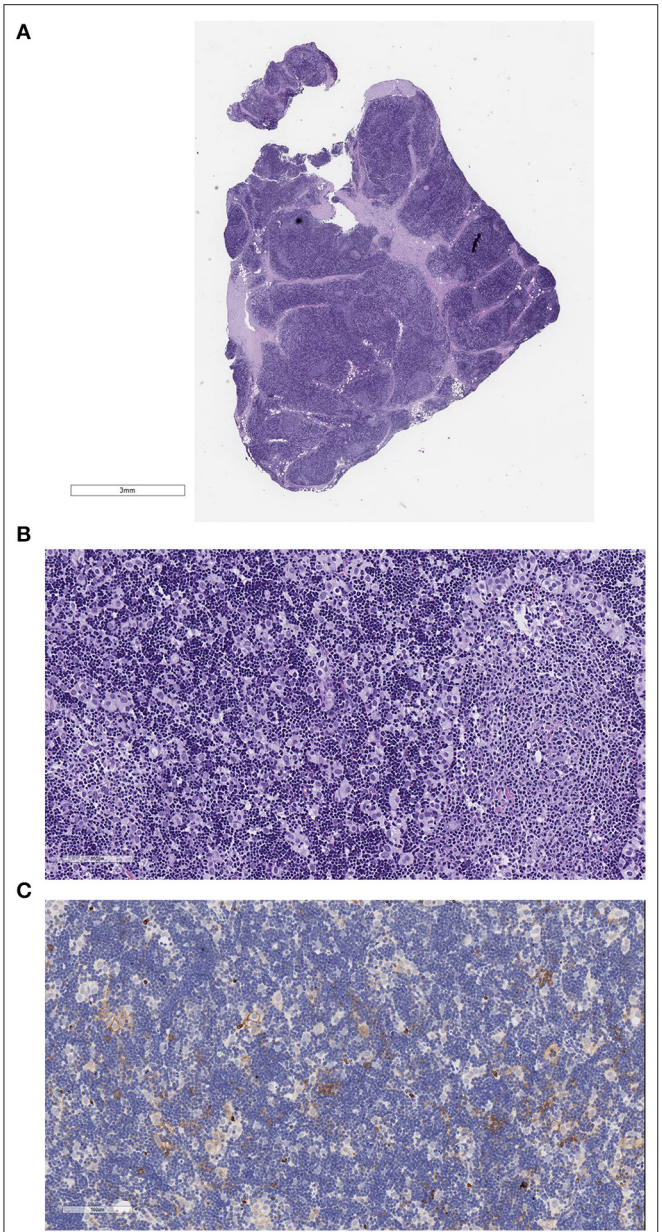

FIGURE 2 | Example of a B2 thymoma fixed in formalin at 4°C and embedded in paraffin. The slides were scanned with the Aperio system 40×. Good morphological details are observed. **(A)** Hematoxylin–eosin (HE) stain, low magnification to show the whole section present on the scanned slide. **(B)** HE stain, 200×, showing the cortex-like tumor rich in epithelial cells (EC) and in thymocytes and a medullary island mostly containing lymphocytes. **(C)** Glut-1 stain of the B2 thymoma. Only few epithelial cells react.

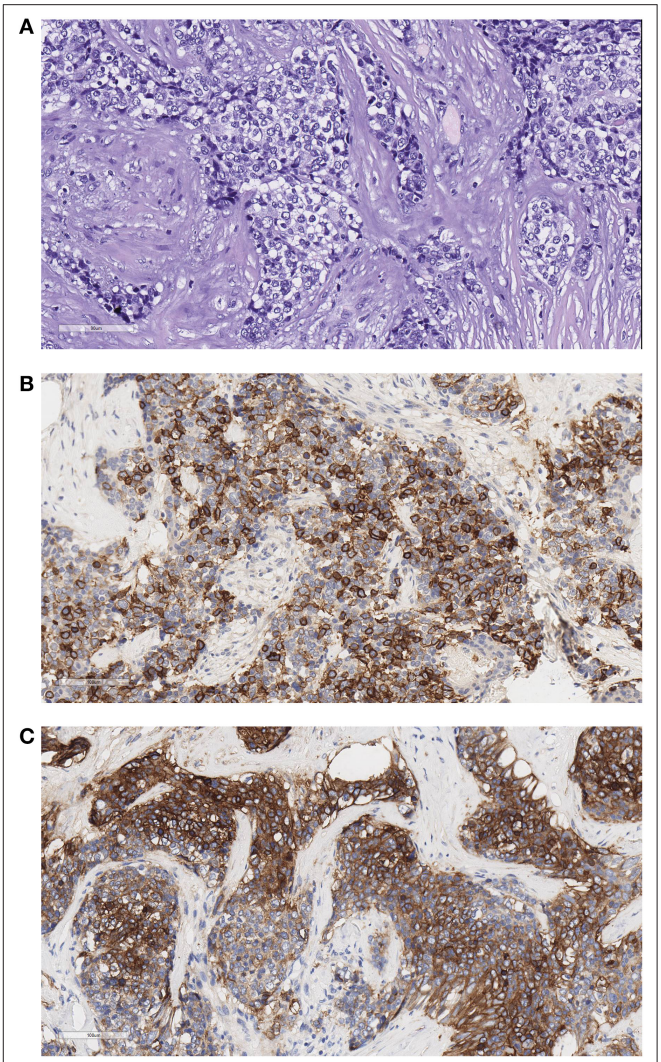

FIGURE 3 | Example of a thymic carcinoma fixed in formalin at 4°C and embedded in paraffin. The slides were scanned with the Aperio system 40×. Good morphological details are observed. **(A)** Hematoxylin–eosin (HE) stain, 200×, showing the atypical epithelial cells forming ribbons infiltrating sclerotic tissue. **(B)** CD117 stain, 200×. Most cells are stained with this thymic carcinoma marker. **(C)** Glut-1 stains in thymic carcinoma ribbons and networks of epithelial cells.

Subsequently, we established a successful collaboration with our Oncogenomic and Epigenetic Unit together with the Sapienza University of Rome, where we approached the epigenetic control of TET by microRNA-focused studies. First, we approached this field by using FFPE materials (43); then circulating microRNAs were investigated (54); subsequently, we contributed high-quality biobank-derived frozen material, allowing the gene expression profile of the mRNA putative target of miR-145-5p (19). We also started to perform the functional characterization of the 1889c cell line (60) by investigating the epigenetic regulation of miR-145-5p, as well as the modulation of its functional target mRNAs in our system. Of note, we are now engaged in the characterization of the contribution of the long non coding RNA (lncRNA) function in TET. Very few reports so far investigated lncRNA in TET (69). We are focusing our attention on the sponge activity of lncRNAs, which are able to inhibit the microRNA function generating molecular networks relevant for tumor establishment and progression. Our preliminary data (not shown) highlight the relevance of the epigenetic deregulation of ncRNA in TET for the identification of novel molecular targets of therapy.

The quality of our biobank material was also confirmed by the inclusion of our samples among the cases included in the TCGA-THYM study (57). Recently, we have focused on implementing our biobanking activities. These were supported by a strategy based on a positive feedback cycle between the thoracic surgeon and the "dedicated" pathologist, by the development of an efficient and certified biobanking system, and by the implementation of laboratory cell culture facilities. In fact, our purpose now is to set up a procedure for the isolation of stem cells from fresh TET specimens, based on our previous experience in different tumor systems (70). Preliminary data on primary cultures of TET appear to be promising (data not shown). In the field of imaging analysis, digital pathology is a rapidly evolving and increasingly utilized tool in histology. It enables high throughput and precise analysis of a large number of samples and facilitates easier interactive consensus in remote diagnostic discussions, as we achieved in the TCGA-THYM study (57). TCGA deriving image archives—otherwise underutilized—recently provided insight into the tumor-immune microenvironment in 13 TCGA tumor types (71). All the studies reported a major role played by the "dedicated" pathologist. The role of pathologist evolved from giving microscopic description to adhering to internationally validated classification criteria (38) and to adopting structured pathology reports (37) in order to provide standardized and relevant information for prognostic stratification of patients. The pathologist also plays a major role in identifying new biomarkers by IHC; digitized slides provide quantitative as well as qualitative observations. Moreover, the morphological evaluation of tumor samples for molecular analyses prevents inadequate sampling and inappropriate molecular analyses on necrotic or fibrotic tissue. Bridging the gap between molecular data and the knowledge of the biological/tumoral systems, the pathologists contribute to integrating morphology with molecular findings. Based on our examples above, it is evident that solid commitment from the Pathology Department is critical for translational research and in all aspects of clinical care, especially in rare tumor types.

The challenging points of our well-established study on TET and of tissue-based translational studies range from the limited availability of cases and funding to the difficulties in clinical data collection. Moreover, given the specific biology of TET, outcome indicators are difficult to collect due to the long natural history of thymomas and to the possibilities of patients migrating or returning to their place of origin, being lost to follow-up. Clinical trials for TET (48) are difficult to promote and to find collaborative support from pharmaceutical companies, as these tumors are orphan diseases (10). Currently, at our institute, new TET cases are discussed at our multidisciplinary thoracic tumor Board meetings (49, 72) as they are an important tool in achieving the best approach to patient management. Our Institute routinely performs second opinion pathological review for the majority of patients who seek oncologic consultations. A second look in specialized centers for rare tumors can result in major prognostic and therapeutic modifications (73). Despite the limited funding for our translational research projects on TET, we have received free support from our research collaborating units who have contributed in providing reagents, human resources, and the use of their platforms. This type of eager collaborative support happens when there is a deep-seated belief in a type of rare tumor that is deserving of attention and interest. At the same time, health networks such as EURACAN provided improvement in patient assistance (74) and are expected to promote translational research in rare tumor.

Therefore, although our clinical responsibilities have been greatly burdened over the last few years, we, as a team, have set the grounds for significantly contributing scientifically to TET research. We hope to implement our translational research activity by improving our networking with other research centers in both Italy/Europe and abroad. In the future, translational research will offer precision medicine data and targeted therapies to the clinical management of TET patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of our Ethical Committee. The protocol was reviewed and approved by the Comitato Etico Centrale IRCCS Lazio -Found. Bietti. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MM designed the manuscript and drafted it. EM, EG, SM, FG, VL, GA, and FFaz participated in the designing and drafting up of the manuscript. GB and NG critically revised it. EP, FFac, LC, and GC coordinated the manuscript. Administrative support was given by EP. All the authors contributed to the work during the years by their clinical or experimental activity. All authors contributed to the article and approved the submitted version.

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Influence of Intratumor Heterogeneity on the Predictivity of MGMT Gene Promoter Methylation Status in Glioblastoma

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Brigliadori G, Goffredo G, Bartolini D, Tosatto L, Gurrieri L, Mercatali L and Ibrahim T (2020) Influence of Intratumor Heterogeneity on the Predictivity of MGMT Gene Promoter Methylation Status in Glioblastoma. Front. Oncol. 10:533000. doi: 10.3389/fonc.2020.533000

Glioblastoma is the most aggressive tumor of the central nervous system. Prognosis is poor, even in the presence of a methylated state of MGMT gene promoter, which represents the biomarker with the highest prognostic/predictive value for the standard treatment of patients. Among patients with a methylated MGMT status, we identified an intermediate range of methylation above the standard 9% cut-off (gray zone) in which the predictive strength of the marker was lost. In an effort to improve the evaluation of the biomarker in clinical decision-making, we are carrying out a retrospective study, performing an in-depth analysis of samples used for diagnosis to understand how molecular heterogeneity, a hallmark of glioblastoma, impacts the evaluation of MGMT gene promoter methylation. Preliminary data from samples belonging to the "gray zone" tend to confirm the hypothesis of a mismatch between methylation values used for clinical decision-making and those included in our in-depth analysis. Confirmation of these data would help to better define the predictive power of MGMT promoter methylation status and greatly facilitate clinical decision-making.

Keywords: temozolamide, MGMT methylation, intratumor heterogeneity, predictivity, glioblastoma

Among brain tumors, glioblastoma (grade IV according to World Health Organization) is the most aggressive form of disease, with an average survival ranging from 12 to 15 months (1, 2). Currently, only a small number of molecular markers are recognized in brain diseases compared to other cancers. One of the molecular markers with the highest prognostic/predictive impact in glioblastoma is the methylation status of the promoter of the O_6-methylguanine DNA methyltransferase (MGMT) gene, which encodes for an enzyme involved in the DNA repair system. Standard treatment for glioblastoma is the "Stupp protocol" (3), comprising radiotherapy and chemotherapy with the alkylating agent temozolomide (TMZ). When the MGMT promoter is in a "methylated" state, a better response to the treatment is expected.

The main issues relating to the evaluation of the degree of methylation of the MGMT promoter are as follows:

- There is no cutoff value that uniquely discriminates between methylated and unmethylated states. Using the quantitative pyrosequencing method, the current reference value is 9% (4).
- A precise evaluation of the prognostic/predictive value of the degree of methylation is still a matter of debate.

We carried out a retrospective study aimed at defining a methylation cutoff value, identifying a value of 30% methylation as discriminant between the methylated and unmethylated state (5). Of note, we found that patients who underwent the same type of surgery (radical or non-radical) and had a MGMT methylation value ranging from 10 to 29% showed a poorer overall survival (OS) than those with unmethylated MGMT (9.8 vs. 19.5 months, respectively). Starting from this observation, we decided to perform an in-depth evaluation of this subset of patients in whom the predictive power of the marker is lost, calling the methylation range (10–29%) in question the *gray zone*.

Given the well-known molecular heterogeneity of the tumor, we hypothesize that the mismatch with the predictive value of the marker could be due to misinterpretation of the methylation status. Several studies are currently underway to investigate the clinical/biological impact of this tumor characteristic, which includes MGMT promoter methylation, and differ mainly in their approach to the problem:

- Primary cell culture models isolated from neoplastic lesions (6)
- Molecular analysis on bioptic sections of different areas of the tumor mass (7)
- Assessment of methylation variability of the individual CpG islands within a methylation profile (8).

Although all of these approaches can improve our understanding of the molecular heterogeneity of glioblastoma, their impact on diagnostic decision-making requires further investigation.

We decided to evaluate intratumor heterogeneity in single formalin-fixed paraffin-embedded (FFPE) tissue samples used for diagnosis, using identical subsections of each sample to improve the analysis and to obtain a more accurate evaluation of the methylation status of the MGMT promoter. To this end, we are carrying out a retrospective study on a set of samples from 120 patients with a follow-up of at least 2 years, stratified into four groups of 30 patients each (**Figure 1A**):

a) Non-methylated (0–9%)
b) Low methylated (10–17%)
c) Medium methylated (18–29%)
d) Highly methylated (30–100%).

Two FFPE sections are used for each sample. The DNA of the entire lesion is extracted from one section, whereas the other section is further divided into four subsections, each subjected separately to DNA extraction. Given the impossibility of using a histological basis to subdivide samples, sections are arbitrarily selected, and their homogeneity evaluated by quantifying the amount of DNA obtained in an equal volume of elution buffer.

In a preliminary analysis, we found that the degree of MGMT promoter methylation was fairly similar in some cases, whereas in others it showed considerable variability, with high values in one section and much lower values in others. According to our study design, it is reasonable to assume that, if the methylation value of the entire sample is >30%, there will be a homogenously high methylation in the subsections. Similarly, a uniformly low methylation is expected in subsections when the overall methylation value is <9%. The greatest heterogeneity is expected when the average methylation of the entire sample falls within the gray zone (10–17% and 18–29%).

PRELIMINARY RESULTS

Ten samples were analyzed. Eight samples were within the gray zone: six showing medium methylation (21, 22, 23, 24, and 29%) and two low methylation (each 15%). One sample was in unmethylated state (2%), and one was highly methylated (43%).

As expected, the highly methylated (>30%) and unmethylated (<9%) samples (**Figure 1**) showed subsection methylation values within the considered range. The unmethylated sample (**Figure 1B**) fell into the narrowest range (0–9%) and thus showed the greatest uniformity. The sample with an average 43% methylation (**Figure 1C**) had three out of four sections with methylation >30%, and only one section with a lower value (24%), which was still fairly high with respect to the standard cutoff of 9%. The red line indicates the average methylation of the entire sample.

Considerable variability in the methylation values of single sections was observed in samples belonging to the gray zone. This variability was also present in low methylation samples (**Figures 2A,B**), in which sections showed differences of at least three percentage points with respect to the mean value of the entire sample. In one case (**Figure 2B**, sample section 4), a value fell into the unmethylated range, which, given the narrow range of values (10–17%), would seem to confirm molecular heterogeneity.

Differences between single sections were more marked in samples with medium methylation. In particular, mean methylation values of 29 and 24% (**Figures 1D,E**) could lead to an incorrect evaluation of methylation status. In the former (**Figure 1D**), there was high methylation (47%) in one subsection and low methylation (17%) in the second, whereas the remaining subsections were unmethylated (4 and 1%). Thus, compared to the methylation value that would place the sample in the methylated category, 50% of the sample was unmethylated, and 25% was low methylated. In the latter sample (**Figure 1E**), in contrast to the previous case, 50% of sample subsections showed higher than average methylation values (41 and 31% vs. 24%). Thus, paradoxically, the apparently less methylated sample was actually more methylated. Considering the two samples, the seemingly more highly methylated one was, in fact, the least methylated.

In the other medium methylated samples (**Figures 2C–F**), the differences between the subsections and the entire sample were less remarkable. However, there was always at least one section in each sample showing a methylation value that definitely deviates from the average.

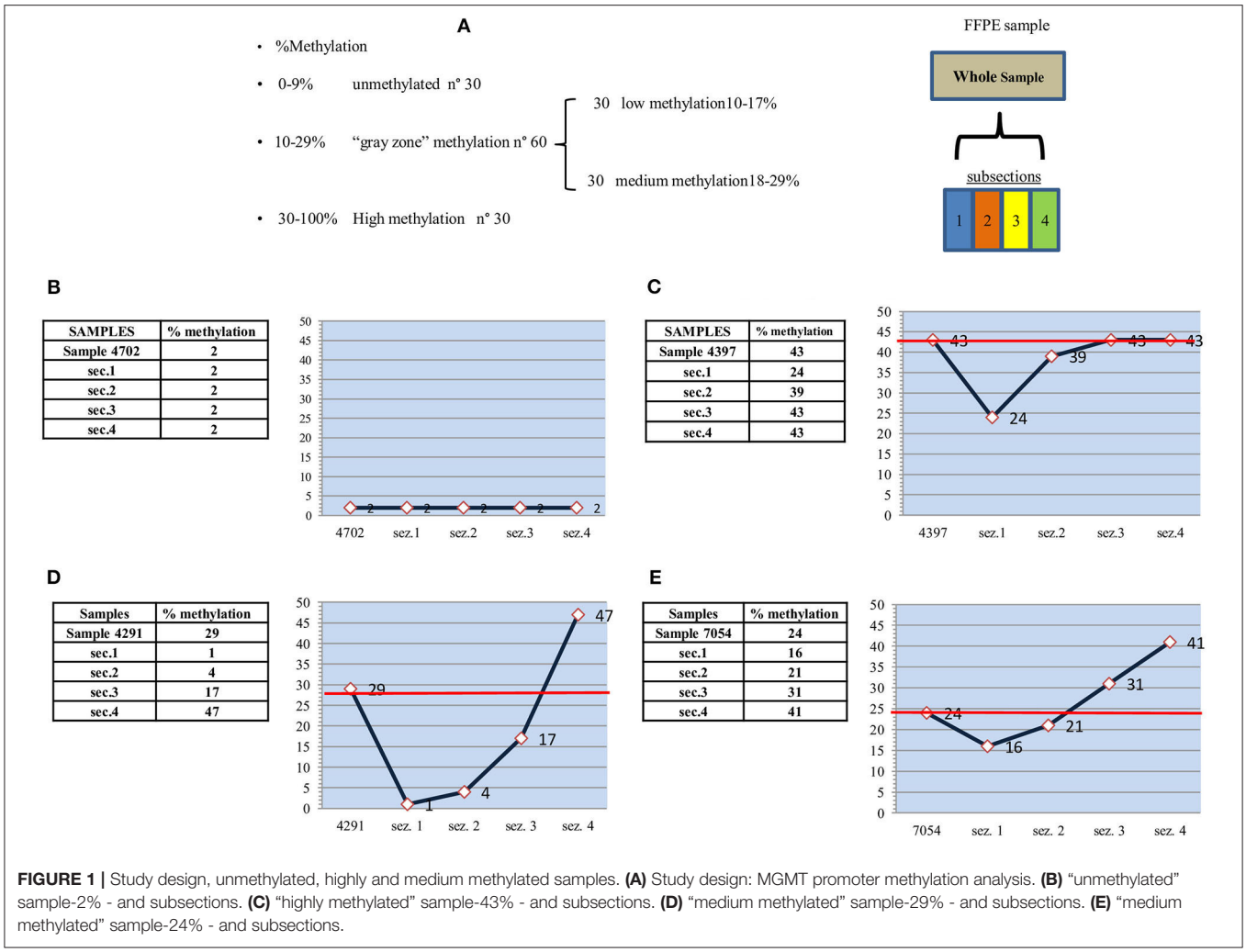

FIGURE 1 | Study design, unmethylated, highly and medium methylated samples. **(A)** Study design: MGMT promoter methylation analysis. **(B)** "unmethylated" sample-2% - and subsections. **(C)** "highly methylated" sample-43% - and subsections. **(D)** "medium methylated" sample-29% - and subsections. **(E)** "medium methylated" sample-24% - and subsections.

DISCUSSION

Although great efforts have been made to improve the outcome of patients with glioblastoma, it remains the leading cause of death among brain tumors, with a dismal prognosis (2). Surgery is the mainstay of treatment, and the Stupp protocol (radiotherapy and chemotherapy with TMZ) (3) represents the only postsurgery treatment obtaining a benefit in either progression-free survival or OS (9–12). The efficacy of TMZ is mainly related to MGMT promoter methylation status, which represents the only prognostic/predictive marker for these patients. However, there are many unanswered questions about the role of MGMT methylation status in patient outcome, its cutoff threshold, and predictive strength. Our previous investigation of these issues led us to propose a 30% methylation cutoff (4). In the same study we identified a subset of patients in which the predictivity of the marker was lost, calling this MGMT methylation range (10–29%) the "gray zone." We hypothesize that this loss of predictivity could be influenced by the molecular heterogeneity of the disease. We are therefore performing a retrospective study to investigate the correlation between the intratumor heterogeneity of MGMT promoter methylation and patient outcome. Our preliminary data appear to confirm the well-known histological heterogeneity of the disease at the molecular level and indicate the need for a more in-depth evaluation of samples belonging to the gray zone.

With regard to the prognostic/predictive value of the marker, we hypothesize that molecular heterogeneity may influence its clinical evaluation, especially in cases that fall within the gray zone. Our preliminary data appear to confirm this because gray zone samples showed an internal methylation distribution that differed significantly from the mean value used for the diagnostic referral. For example, in one case with a relatively high mean methylation (29%) for the entire sample, the value was mainly due to a single section with a very high methylation status (47%), whereas much of the sample showed low (17%) or no methylation (**Figure 1D**). Conversely, a moderately high mean methylation (24%) in another sample had a methylation distribution in which two of the four sections showed values higher than the entire sample

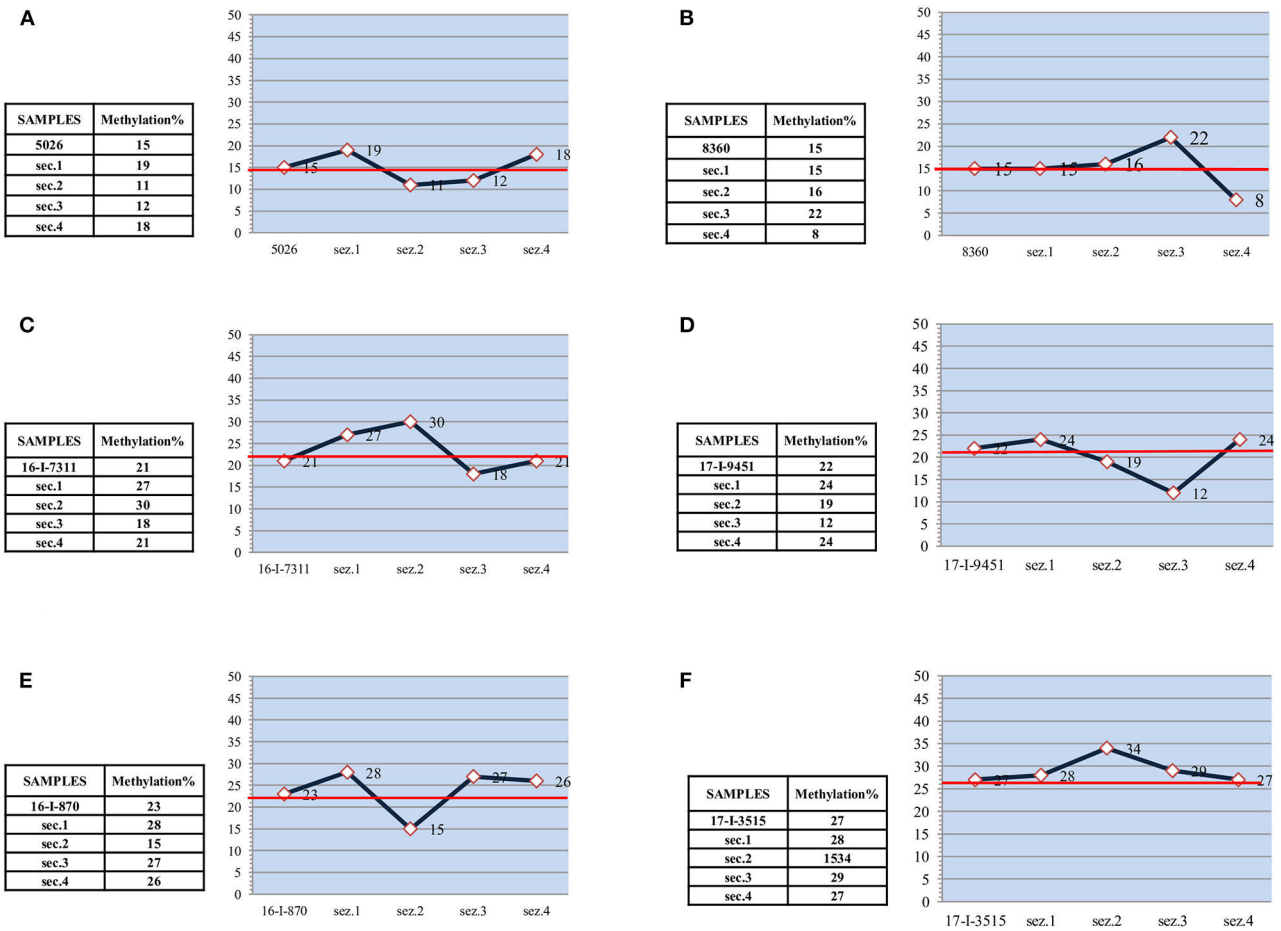

FIGURE 2 | Low and medium methylated samples. **(A)** "low methylated" sample-15% - and subsections. **(B)** "low methylated" sample-15% - and subsections. **(C)** "medium methylated" sample-21% - and subsections. **(D)** "medium methylated" sample-22% - and subsections. **(E)** "medium methylated" sample-23% - and subsections. **(F)** "medium methylated" sample-27% - and subsections.

(31 and 41%) (**Figure 1E**). Consequently, most of the sample was more highly methylated than the mean value used for clinical decision-making. Moreover, intratumor heterogeneity was well-represented, albeit to a lower degree, in all the other samples belonging to the gray zone.

CONCLUSIONS

The small number of samples analyzed is probably the most important limitation of the present study. Despite this, we believe that the heterogeneity found in MGMT promoter methylation values provides sufficient evidence to warrant further investigation. We intend to complete the study with data on patient follow-up and analysis of all cases and will carry out a more in-depth analysis of the samples in which MGMT promoter methylation falls into the gray zone to enhance the prognostic/predictive capacity of the marker and facilitate treatment decision-making.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The protocol was approved by the IRST Medical Scientific Committee, approval no. 5870/5.3, and performed according to Good Clinical Practice standards and the Declaration of Helsinki. All patients gave written informed consent to take part in the study.

AUTHOR CONTRIBUTIONS

GB and GG performed the analyses and drafted the manuscript. DB prepared and selected the FFPE samples. LT performed the surgery. LG selected patients suitable for the study. LM reviewed the manuscript for important intellectual content. TI selected

patients and reviewed the manuscript for important intellectual content. All authors contributed in equal part to the study design, read and approved the final version of the manuscript for publication.

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