ADVANCES IN CANCER STEM CELL BIOLOGY

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ADVANCES IN CANCER STEM CELL BIOLOGY

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EDITORIAL
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Editorial: Advances in Cancer Stem Cell Biology

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Editorial on the Research Topic

Advances in Cancer Stem Cell Biology

The World Health Organization (WHO) reports that an estimated 9.6 million people died from cancer in 2018 (Bray et al., 2018). This estimate includes patients who had a diverse range of different types of cancers, including those arising in the lung, large intestine, stomach, liver, and breast cancer. For all of these tumors, the standard treatment options are surgery, radiotherapy, and chemotherapy. Several factors may influence the prognosis of a cancer patient. One particular factor that correlates with patients' survival is related to the biology of the tumor mass, i.e., whether the tumor grows slowly, fast, or has the capacity to relocate (Zhang et al., 2020; Thurmaier et al., 2021). The biologies of the different types of cancers are at the core connected through the abnormalities of 10 cellular pathways known as the hallmarks of cancer (Hanahan and Weinberg, 2011). Deregulation in these pathways is correlated with chemo and radio resistance (Buckley et al., 2020). A particular pathway that involves sustaining proliferative signaling and enabling cancer cells to behave similarly to embryonic stem cells has been of great interest in the area of translation oncology. Single-cell analysis of different cancers has shown clearly the existence and the diversity of a stem cell program in many tumor cells (Patel et al., 2014; Filbin et al., 2018). Targeting the diverse types of Cancer Stem Cells (CSCs) in *IDH*-wildtype Glioblastoma Multiforme (GBM) using combination therapy has been shown to be synergistic (Wang et al., 2019). Thus, characterizing the properties of CSCs is critical to improving future CSCs-targeting therapies. However, whether the activation of a deregulated stem cell program in CSCs is transient or stable remains to be addressed (Neftel et al., 2019).

The Advances in Cancer Stem Cell Biology topic aimed to provide a recent overview on the molecular biology of CSCs. Different approaches were used in published manuscripts from theory to bioinformatics and to experiments.

Using bioinformatics tools, Sang et al. aimed at identifying markers for CSCs that correlate with immune infiltrates in hepatocellular carcinoma (HCC) and poor patient survival. They utilized the Oncomine database, Gene Expression Profiling Interactive Analysis (GEPIA), and Integrative Molecular Database of Hepatocellular Carcinoma (HCCDB) to analyze the expression of hepatocellular CSC (HCSC) markers in 364 liver cancer samples. The correlation of HCSC markers to tumor-infiltrating immune cells was tested by Tumor Immune Estimation Resource (TIMER). Out of 10 differentially deregulated HCSC markers, 3 (*CD24*, *SOX9*, and *SOX12*) were highly expressed and had a positive correlation with poor prognosis. In contrast, the expression of *CD13*, *CD34*, and *ALDH1A1* was associated with prolonged overall survival. The authors noted that *SOX12* in particular might constitute a therapeutic target for hepatocellular carcinoma. Complementary to

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that work is the Li and Zhu manuscript, which reviewed recent advances in experimental studies on liver CSCs. They showed an update on the latest advances in experimental studies on non-coding RNAs (ncRNAs), oncogenes, and oncoproteins, with a particular focus on three pathways: the Wnt/β-catenin signaling pathway, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, and interleukin 6/Janus kinase 2/signal transducer and activator of transcription 3 (IL6/JAK2/STAT3) signaling pathway. Known associated roles for more than 30 CSC-related genes were discussed in detail. In particular, they conclude that octamer 4 (*OCT4*) and *NANOG* are important functional genes that play a pivotal role in liver CSC regulation and HCC prognosis.

Another bioinformatics-based paper, published by Tian et al., applied a weighted gene co-expression network analysis on gene expression data sets from head and neck squamous cell carcinomas (HNSCC) to define an mRNA expression-based stemness index consisting of genes that served as prognostic markers. Raw data for 643 samples were downloaded from the Cancer Genome Atlas (TCGA) database and the Gene Expression Omnibus (GEO) website. The study showed that the combined deregulated expression of eight stem-cell-related markers (*RGS16, LYVE1, hnRNPC, ANP32A, A1MP1, ZNF66, PIK3R3*, and *MAP2K7*) has a powerful capacity for overall survival prediction. They support their bioinformatics data by detecting the level of expression in HNSCC cell lines. The authors concluded that their proposed model could contribute to a better understanding of the role of HNSCC stem cells in developing targeted therapy.

An experimentally based approach was presented in the manuscript authored by Li et al. This work investigated the association of SET Domain Containing 2 (*SETD2*) gene mutations/variants with clinical features and prognosis in patients with Myelodysplastic syndrome (MDS). SETD2 is a transcriptional regulator and has been previously shown to be required for the self-renewal of hematopoietic stem cells (HSCs), and SETD2-deficient HSCs were shown to contribute to the development of MDS. Using targeted next-generation sequencing, the results indicated that out of 203 patients with MDS, 37 patients had *SETD2* gene mutations/variants, and these patients exhibited a significantly increased frequency of *TP53* mutations. Low expression of *SETD2* in patient tumor cells was identified as a risk factor for progression-free survival (PFS). The study concluded that *SETD2* deficiency contributes to genomic instability and is associated with unfavorable prognosis in patients with myelodysplastic syndrome.

Three more review articles were published in this collection. The first was a mini review by Azzarelli, which discussed the emerging 3D models of glioblastoma that overcome certain limitations of monolayer cultures. The author concluded that glioblastoma brain organoids provide the opportunity to study CSC lineages and serve as tools to predict tumor progression and treatment response. In a second review, Xu et al. discussed the role of N6-methyladenosine (m6A) in the differentiation of CSCs. The authors highlighted that targeting m6A modification of CSCs constitutes a yet not fully explored option for drug treatment of cancer. The third review was presented by Alhabbab, and it described how CSCs employ various mechanisms to modulate the immune system response. The review outlined the recent knowledge for the interactions between CSCs' common markers, including CD133, CD90, EpCAM, CD44, ALDH, and EGFRVIII, and the immune system. Current information on CAR T cell genetic engineering and signaling, CAR T cells, and the barriers in using CAR T cells as immunotherapy to treat solid cancers in the context of targeting CSCs were detailed.

Finally, in a theory-based article, Manzo investigated the nature of tumor growth within a mathematical model, which assumes tumors encompass CSCs that behave similarly to para-embryonic stem cells and divide into a hierarchic sequence of CSCs and non-CSCs. Tabulating theoretical data using this model, the author identified defined mathematical relationships between CSCs and non-CSCs that were similar to experimental data. The model explains tumor progression in a modular way that recalls the propagation of tumor spheres *in vitro*. Furthermore, the author discussed similar features, including nature form, dimension, cell distribution, and layer compartmentation for avascular tumors, tumor spheres, and preimplantation blastocysts. The author concluded that the presented mathematical model provides further support for the para-embryonic nature of the cancer process.

The research on CSCs is ongoing, and several concepts still remain to be addressed or fully explained. For example, what combinations of markers define different types of CSCs, and how does the "combined markers identity tag" correlate with therapeutic prognosis? Perhaps next-generation single-cell sequencing in combination with multiplex protein array technology could shed more light on the characteristics of CSCs and CSC markers. A unified CSC-specific interactive database for the mutational signatures and genomic instability of CSCs is likely to improve cancer research. Some questions remain: How do CSCs contribute to metastasis, and what are the therapeutics that can be given to combat CSCs movement and colonization? How can the gene expression profile of CSCs be stabilized and prevented from shifting in response to the microenvironment? What are the clinically relevant CSCs models that provide highly efficient translational protocols that can be utilized in a clinical setting?

Taken together, the variety of the authors' topic contributions, either by focused reviews, theoretical considerations, or research articles, has shed light on current advances in CSC biology and support further approaches for integrative CSC research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Human Hepatic Cancer Stem Cells (HCSCs) Markers Correlated With Immune Infiltrates Reveal Prognostic Significance of Hepatocellular Carcinoma

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Background: Several markers have been reported to be specific for hepatic cancer stem cells (HCSCs), which is usually thought to be highly associated with poor clinical outcomes. Tumor-infiltrating immune cells act as an important factor for oncogenesis. Little is known about the correlation of HCSC markers to prognosis and immune infiltrates.

Methods: Expression of HCSC markers was analyzed through Oncomine database, Gene Expression Profiling Interactive Analysis (GEPIA) and Integrative Molecular Database of Hepatocellular Carcinoma (HCCDB), respectively. The prognostic effect of HCSC markers was evaluated using Kaplan-Meier plotter in association with different tumor stages, risk factors, and gender. The correlation of HCSC markers to tumor-infiltrating immune cells was tested by Tumor Immune Estimation Resource (TIMER). HCSC markers related gene sets were investigated by GEPIA, with their biological functions being analyzed by Cytoscape software.

Results: The expression level of 10 HCSC markers in HCC was higher than that in normal tissues in at least one database. Among them, high expression of *CD24*, *SOX9,* and *SOX12* was positively correlated with poor prognosis (*CD24*: OS P = 0.0012, PFS P = 7.9E–05. *SOX9*: OS P = 0.012. *SOX12*: OS P = 0.0004, PFS P = 0.0013, respectively). However, the expression of *CD13*, *CD34* and *ALDH1A1* was associated with prolonged OS and PFS. *SOX12* was significantly upregulated in poor prognosis of HCC patients with different conditions. Besides, total nine HCSC markers were identified to be positively associated with immune infiltration, including *SOX12*. Furthermore, Toll-like receptor signaling pathway was found to be one major pathway of these HCSC markers related gene networks.

Conclusion: Our results suggest that seven upregulated HCSC markers (*CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9, CK19,* and *SOX12*) are related with poor prognosis and immune infiltration in HCC. In addition, we find that high *SOX12* expression remarkably affect prognosis in male HCC patients but not in female. HCC patients under viral infection or

alcohol intake with increased *SOX12* expression had poorer prognosis. Therefore, HCSCs markers likely play an important role in tumor related immune infiltration and *SOX12* might be a potential therapeutic target in patients with HCC.

Keywords: cancer stem cell, hepatocellular carcinoma, prognostic biomarker, immune infiltrates, hepatocellular carcinoma

INTRODUCTION

Liver cancer is the second leading cause of worldwide cancer death in men, and sixth in women (Torre et al., 2015; Ferlay et al., 2019), and it accounts approximately 50% of the total number of cancer cases and deaths in China (Torre et al., 2015). The most common liver cancer (~78%) is hepatocellular carcinoma (HCC), the primary malignant neoplasm derived from hepatocytes (Laursen, 2014; Zhu et al., 2016). It has been known that the tumor-infiltrating immune cells play a key role in tumor microenvironment of HCC, such as tumor-associated macrophages (TAMs) (Werb and Coussens, 2002) and tumor-infiltrating lymphocytes (TILs) (Chen and Mellman, 2013). TAMs produce factors that maintain cancer-related inflammation and potentiate tumor progression (Schoppmann et al., 2002), whereas some TILs may control cancer outcome (Gao et al., 2007). So far emerging immunotherapies of immune checkpoint blockade for HCC, like programmed death-1 (PD-1) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), are still in the start-up stage compared to other tumors. And the objective response rate to the anti-PD-1 and anti-CTLA-4 treatment is relatively low (Johnston and Khakoo, 2019). Due to the immune-suppressive microenvironment of HCC, new checkpoint blockade inhibitors or combining checkpoint blockade inhibitors with other methods may be needed to reinforce the effect (Prieto et al., 2015). Therefore, it is urgent to clarify tumor-immune interactions and identification of novel immune-related therapeutic targets in HCC.

Hepatic cancer stem cells (HCSCs) are small populations of stem-like hepatocarcinoma cells which has capacity to initiate and maintain HCC growth (Wang et al., 2018). Recent advances of HCSCs have enabled the identification of cell surface protein markers, showed their characteristics of oncogenicity, metastasis and therapeutic resistance. CD133 (PROM1) was first proposed to be a specific HCSC marker in 2006 (Suetsugi et al., 2006). After that, others were identified, including CD90 (THY1), epithelial cell adhesion molecules (EpCAM), CD24, CD13 (ANPEP), CD34, sex determining region Y-box 9 (SOX9), ATP-binding cassette, subfamily G, member 2 (ABCG2), CD44, aldehyde dehydrogenase (ALDH), CK19 (KRT19), sex determining region Y-box 12 (SOX12), and CD47 (Ma et al., 2008; Yang et al., 2008; Yamashita et al., 2009; Haraguchi et al., 2010; Lee et al., 2011; Zhang et al., 2013; Fernando et al., 2015; Kawai et al., 2015; Park et al., 2015; Li W. et al., 2017; Richtig et al., 2017; Zou et al., 2017; Rodríguez et al., 2018; Wang et al., 2019). Various HCSC markers correlate with diversified forms of cells. Several studies demonstrated that there were different phenotypes of HCSCs in one single HCC specimen with polymorphic cellular features and tumorigenic potentials (Yamashita et al., 2013; Ho et al., 2019), indicating the complexity of HCSCs. Thus, the characteristics and regulatory mechanisms of HCSCs are not fully elucidated.

A better understanding of immune-related mechanism of HCSCs may help to find novel HCSCs-specific targets for immunotherapy. Unfortunately, this knowledge is limited. Hence, here we comprehensively investigated the expressions of HCSC markers and the correlations with prognosis and immune infiltration of HCC patients based on the online database. Furthermore, we constructed HCSC markers-related gene networks and analyzed the function of the networks using bioinformatics tools. The findings in this report reveal the prognostic role of HCSC makers in HCC, and provide a potential relationship and mechanism between HCSCs and immunity.

MATERIALS AND METHODS

Oncomine Database Analysis

The online database Oncomine (https://www.oncomine.org/resource/login.html) is a bioinformatics analysis tool across 18,000 cancer gene expression microarrays (Rhodes et al., 2007). The expression level of HCSC marker genes in HCC was identified in the Gene Summary view of Oncomine database. The following values: *P*-value of 0.01, fold change of 2, gene rank of top 10%, and data type of mRNA were applied to determine the threshold.

GEPIA Database Analysis

The online database Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html) is a developed interactive website to analyze the RNA sequencing expression data from the TCGA and GTEx projects (Tang et al., 2017). The expression of HCSC marker genes was confirmed by GEPIA in LIHC dataset. The threshold was determined with the following values: *P*-value of 0.01, fold change of 2, and matched normal data of TCGA normal and GTEx data. GEPIA was also used to generate pathological major stage plot, as well as search for genes that has a similar expression pattern with HCSC markers in liver hepatocellular carcinoma (LIHC).

HCCDB Database Analysis

The online database Integrative Molecular Database of Hepatocellular Carcinoma (HCCDB) (http://lifeome.net/database/hccdb) curated 15 public HCC expression datasets to serve as a one-stop online resource for exploring gene expression of HCC (Lian et al., 2018). The expression of HCSC marker genes was confirmed by HCCDB.

Kaplan-Meier Plotter Database Analysis

Kaplan-Meier plotter (liver cancer) is an online platform that can assess the RNA-seq data of 364 liver cancer samples (http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq) (Menyhart et al., 2018). The correlation between expression level of HCSC marker genes and survival in liver cancer was analyzed by Kaplan-Meier plotter. Best cutoff, computed hazard ratio (HR) with 95% confidence intervals and *P* value were selected for the analysis of split patients.

UALCAN Database Analysis

UALCAN is a comprehensive, user-friendly, and interactive web resource for analyzing TCGA transcriptome and clinical patient data (http://ualcan.path.uab.edu/index.html) (Chandrashekar et al., 2017). UALCAN is designed to provide easy access to publicly available cancer OMICS data (TCGA and MET500). In addition, it enables researchers to study the expression level of genes, not only to compare primary tumor with normal tissue samples, but also to compare across different tumor subgroups as defined by pathological cancer stages, tumor grade, gender, and other clinico-pathologic features.

TIMER Database Analysis

Tumor Immune Estimation Resource (TIMER) is a computational tool to investigate gene expression characterization of tumor-immune interactions in more than 30 cancer types (https://cistrome.shinyapps.io/timer/) (Li T. et al., 2017). TIMER is a resource for systematical evaluations of the clinical impact of different immune cells in diverse cancer types. In this study, the correlation between the expression level of HCSC marker genes and the abundance of immune infiltrates in LIHC dataset was analyzed.

Gene Ontology and KEGG Pathway Enrichment Analysis

Gene Ontology (GO) is an internationally-standardized gene functional classification system which offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe properties of genes and their products in organisms. The functional genes were annotated by GO database (http://www.geneontology.org/) using hypergeometric test to examine the biological functions and pathways. GO functional enrichment analysis provides GO terms which are significantly enriched in the functional genes comparing to the genome background, showing which are connected to the wanted biological functions.

Pathway-based analysis helps further understand genes biological functions. KEGG is the major public pathway-related database of biological systems that integrates genomic, chemical and systemic functional information. KEGG provides a basic knowledge for linking genomes to life through the process of pathway mapping. Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in the functional genes comparing with the whole genome background.

In this study, an online biological tool DAVID 6.8 and Clue GO were applied to analyze the molecular and functional characteristics of HCSC markers as well as the related gene expression network.

Statistical Analysis

The KaplanMeier plots was applied to generate survival curves. Subsequently, the outcomes generated from Oncomine were displayed with *P*-values, fold changes, and ranks. HR and *P* or Cox *P*-values from a log-rank test were used to display the results of KaplanMeier plots, and GEPIA. Furthermore, spearman's correlation and statistical significance were applied to evaluate the correlation of gene expression, and the strength of the correlation was determined using the absolute values. *P*-values < 0.05 were considered statistically significant.

RESULTS

Transcriptional Levels of HCSC Markers and Correlation With Pathological Parameters in Patients With HCC

To determine the differences between expression level of HCSC markers in HCC and normal tissues, the mRNA levels of CD90, EpCAM, CD133, CD24, CD13, CD34, SOX9, ABCG2, CD44, ALDH1A1, ALDH3A1, CK19, SOX12, and CD47 in HCC and normal tissues were analyzed based on Oncomine, GEPIA and HCCDB database, respectively. The results from different databases showed to be a little different from each other (**Supplementary Figure 1**). The mRNA expression levels of nine HCSC markers were up-regulated in patients with HCC in Oncomine database, while seven and six were up-regulated in GEPIA database and HCCDB, respectively (**Figures 1A–C**). Among them, *CD90*, *SOX9*, *CD34*, *CD24,* and *ALDH3A1* were significantly increased in all three databases (**Figure 1D**). The *P* value of the five HCSC markers from 12 datasets in HCCDB was listed in **Table 1**.

Moreover, the expression of HCSC markers with tumor major stage of HCC were analyzed by GEPIA. *CD133* ($P = 0.0283$), *CD24* ($P = 0.0132$), *SOX12* ($P = 0.0047$), and *ALDH1A1* ($P = 0.0011$) were significantly varied, respectively, whereas other HCSC markers showed no significant difference (**Figure 1E**, **Supplementary Figure 2A**). To further confirm the results, the expression of these varied genes with different tumor stages were analyzed by UALCAN database. The results indicated that the expression level of *CD24* ($P = 0.0015$) and *SOX12* ($P = 0.0028$) was higher in stage III than that in stage I (**Supplementary Figure 2B**). In addition, the expression level of *SOX12* ($P = 0.0121$) was significantly increased on axillary lymph nodes metastasis compared to no regional lymph node metastasis in HCC (**Supplementary Figure 2C**).

We then asked whether the variation of HCSC markers expression was consistent with the gender since a higher incidence of HCC was shown in men than that in women. As a result, the expression of *ABCG2*, *ALDH1A1,* and *ALDH3A1* was significantly increased in male HCC patient compared to female, but *EpCAM*, *CD24*, *CD13,* and *CK19* showed the

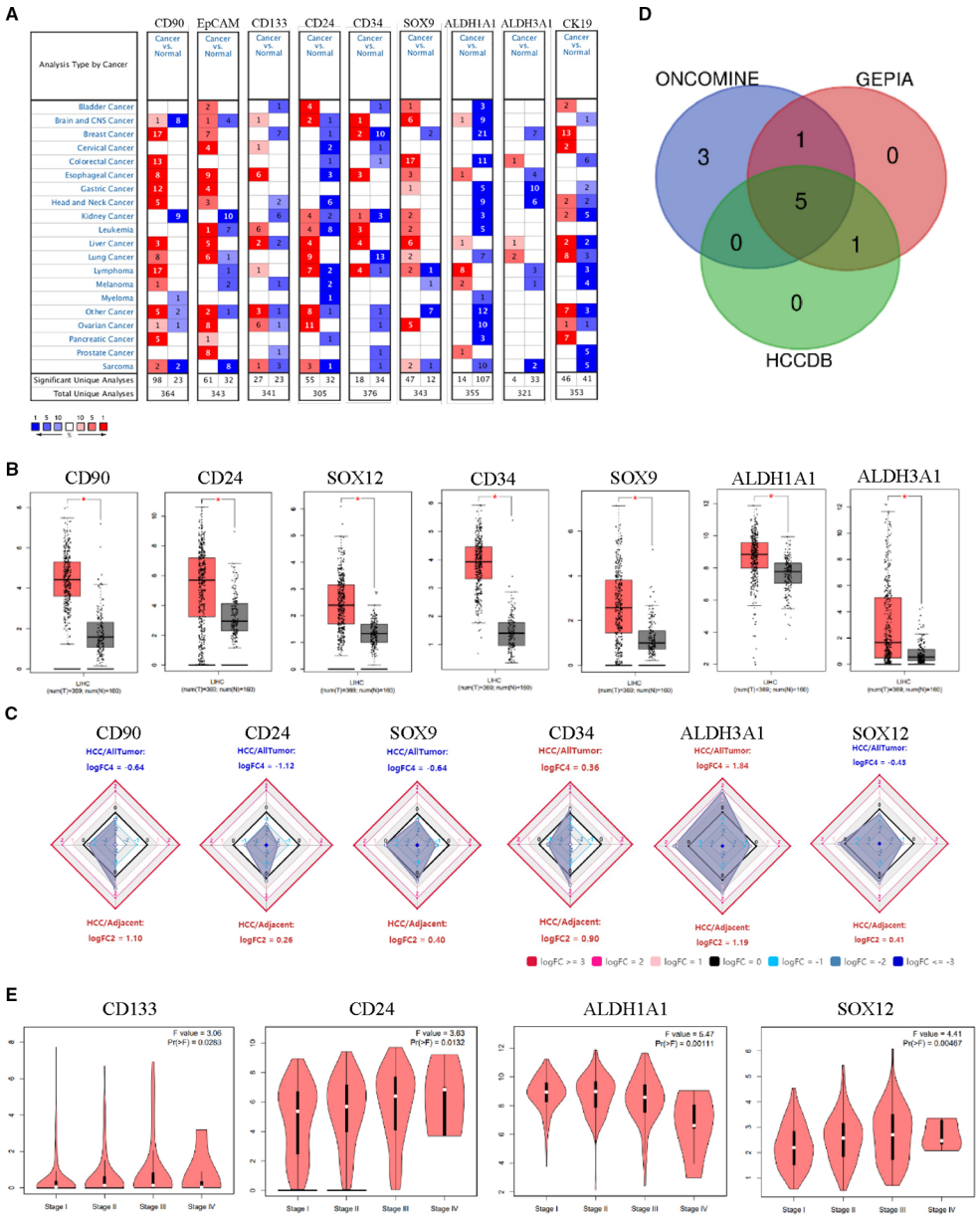

FIGURE 1 | HCSC markers expression levels in HCC. **(A)** Increased HCSC markers in data sets of HCC compared with normal tissues in Oncomine database. Cell color is determined by the best gene rank percentile for the analyses within the cell. **(B)** Increased HCSC markers in data sets of HCC compared with normal tissues in GEPIA. Asterisk: $P < 0.01$. **(C)** Increased HCSC markers in data sets of HCC compared with normal tissues in HCCDB. **(D)** Wayne diagrams of the three database results. **(E)** HCSC markers expression levels at tumor major stages in HCC in GEPIA.

opposite result (**Table 2**). This indicated that gender might be an important factor to influence HCSC markers.

Association of HCSC Markers Expression With Prognosis in HCC Patients

Overall survival (OS) is the period from randomization to death in any cases, which is often considered to be the best end-point of efficacy in cancer clinical trials. Progression-free survival (PFS) refers to the period from randomization to tumor progression or death, reflect both tumor growth, and can be evaluated before confirming the survival benefit. To estimate the influence of HCSC markers expression on prognosis of HCC, the correlation of HCSC markers mRNA expression with OS and PFS of HCC were analyzed using Kaplan-Meier Plotter database. The results of all HCSC markers were shown in **Supplementary Figure 3**. Among them, high expression of *CD13*, *CD34* and *ALDH1A1* was negatively correlated with poor prognosis (*CD13*: OS P = 0.0012, PFS P = 0.0004. *CD34*: OS P = 0.0018, PFS P = 0.003.

TABLE 1 | P value of the five increased HCSC markers in HCCDB database.

Dataset	Source	CD90	SOX9	CD34	CD24	ALDH3A1
HCCDB1	GSE22058	1.17E–26	2.85E–01	1.96E–38	NA	3.05E–06
HCCDB3	GSE25097	5.21E–18	6.57E–07	5.54E–35	NA	2.60E–07
HCCDB4	GSE36376	1.10E–100	6.80E–31	8.70E–36	7.42E-28	3.39E–15
HCCDB6	GSE14520	5.27E–23	5.67E–17	6.63E–36	NA	1.16E–04
HCCDB7	GSE10143	NA	2.73E–01	3.16E–09	NA	8.65E–09
HCCDB11	GSE46444	7.41E–01	3.75E–01	7.56E–01	1.56E–02	1.82E–02
HCCDB12	GSE54236	6.53E–02	1.38E–01	1.63E–11	8.27E–01	1.03E–04
HCCDB13	GSE63898	8.52E–16	1.29E–01	1.30E–03	5.30E–05	7.35E–25
HCCDB15	TCGA-LIHC	5.74E–21	1.10E–02	5.56E–31	1.32E–03	1.07E–05
HCCDB16	GSE64041	2.71E–06	3.48E–01	3.00E–17	6.12E–01	1.58E–06
HCCDB17	GSE76427	1.73E–05	6.89E–03	5.77E–01	1.03E–01	3.17E–01
HCCDB18	ICGC-LIRI-JP	6.33E–46	2.88E–06	2.70E–53	2.90E–10	3.77E–25

TABLE 2 | Expression of HCSC markers in HCC based on patient's gender in UALCAN database.

Gene name	TPM (median)		*P* value
	Male (n = 245)	Female (n = 117)	
CD90	29.145	32.935	0.0802
EpCAM	0.262	0.693	**0.0023**
CD133	0.016	0.022	0.0521
CD24	29.988	83.881	**0.0066**
CD13	151.728	187.890	**0.0003**
CD34	19.673	18.711	0.7202
SOX9	5.439	5.785	0.3324
ABCG2	4.692	2.574	**2.33E-06**
CD44	6.759	5.179	0.1274
ALDH1A1	701.452	400.749	**1.65E-07**
ALDH3A1	2.733	0.435	**0.0024**
CK19	0.486	0.493	**0.0147**
SOX12	5.003	7.082	0.1456

TPM, Transcript per million. Bolded text in p value means statistically significant.

ALDH1A1: OS $P = 0.024$, PFS $P = 0.035$.). On the contrary, high expression of *CD24*, *SOX9,* and *SOX12* was positively correlated with poor prognosis (*CD24*: OS $P = 0.0012$, PFS $P = 7.9E-05$. *SOX9*: OS $P = 0.012$. *SOX12*: OS $P = 0.0004$, PFS $P = 0.0013$.) (**Figures 2A, B**). We also evaluate the effect of HCSC markers mRNA expression level on HCC patient survival by UALCAN database, and obtain the similar results (**Figure 2C**).

From the above results, we noticed that HCSC markers may have two sides on HCC survival, and each of them had different performance. Hence, the correlation of expression of these markers with OS and PFS of HCC in different tumor stages were further analyzed. In the early stages of HCC, high expression of *EpCAM* (OS $P = 0.015$, PFS $P = 0.023$), *CD133* (OS $P = 0.028$), *CD13* (OS $P = 0.0044$, PFS $P = 0.0065$), *CD34* (OS $P = 0.017$, PFS $P = 0.0074$), and *CK19* (OS $P = 0.0067$, PFS $P = 0.014$) had positive correlation with good prognosis, respectively, while *CD44* (OS $P = 0.0087$), *SOX12* (OS $P = 0.033$), and *CD24* (PFS $P = 7.9E-05$) had negative correlation (**Supplementary Figure 4**). Meanwhile, in stage III and IV of HCC, high expression of *ABCG2* (OS $P = 0.02$), *ALDH1A1* (OS $P = 0.042$), *EpCAM* ($P = 0.038$), *CD133* (PFS $P = 0.045$), *CD13* (PFS $P = 0.042$) *CD44* (PFS $P = 0.031$), and *CD47* (PFS $P = 0.011$) had positive correlation with good prognosis, respectively, while *CD24* (OS HR $P = 0.011$), *SOX9* (OS $P = 0.0025$) and *SOX12* (OS $P = 0.0005$, PFS $P = 0.0077$) had negative correlation (**Supplementary Figure 5**).

Considering the effect of gender on prognosis, the correlation of HCSC markers expression with OS and PFS of HCC were evaluated based on patients' gender. Unexpectedly, the high expression of *SOX12* (Male: OS $P = 5.9E-5$, PFS $P = 2E-5$. Female: OS $P = 0.3$, PFS $P = 0.42$) showed close correlation with poor prognosis of male HCC patient, but not the female (**Supplementary Figures 6A, B**). Interestingly, the expression of *SOX12* in the male and female was similar (**Supplementary Figure 6C**, **Table 2**).

High Expression of SOX12 Impacts the Prognosis in HCC Patients With Risk Factors

Alcohol consumption and hepatitis virus are risk factors for HCC (Forner et al., 2018). Unique correlations between HCSC markers and HCC survival rate were found under different risk factors by conducting the analysis in Kaplan-Meier Plotter database as well. *SOX12* was up-regulated in poor OS and PFS with alcohol consumption or hepatitis virus. Besides, *SOX12* (OS $P = 5.2E-06$, PFS $P = 3.3E-05$.) showed more significantly negative correlation with prognosis under alcohol consumption than that under hepatitis virus (**Figure 3C**). *CD24* (OS $P = 0.0028$, PFS $P = 0.0018$) and *SOX9* (OS $P = 0.0023$, PFS $P = 0.013$) both significantly up-regulated in poor OS and PFS in HCC without alcohol consumption and hepatitis virus (**Figure 3A**). *ALDH1A1* (OS $P = 0.025$, PFS $P = 0.026$.) and *ALDH3A1* (OS $P = 0.0016$, PFS $P = 0.0016$.) were specially up-regulated in poor OS and PFS with hepatitis virus (**Figure 3B**).

The above results indicated heterogeneity among these HCSC markers from clinical outcomes. Relationships between different HCSC markers and different tumor stages varied greatly with risk factors of prognosis in HCC. Besides, the effect of same HCSC markers on HCC was different under diverse conditions, suggesting the regulatory function of HCSC markers would be intricate.

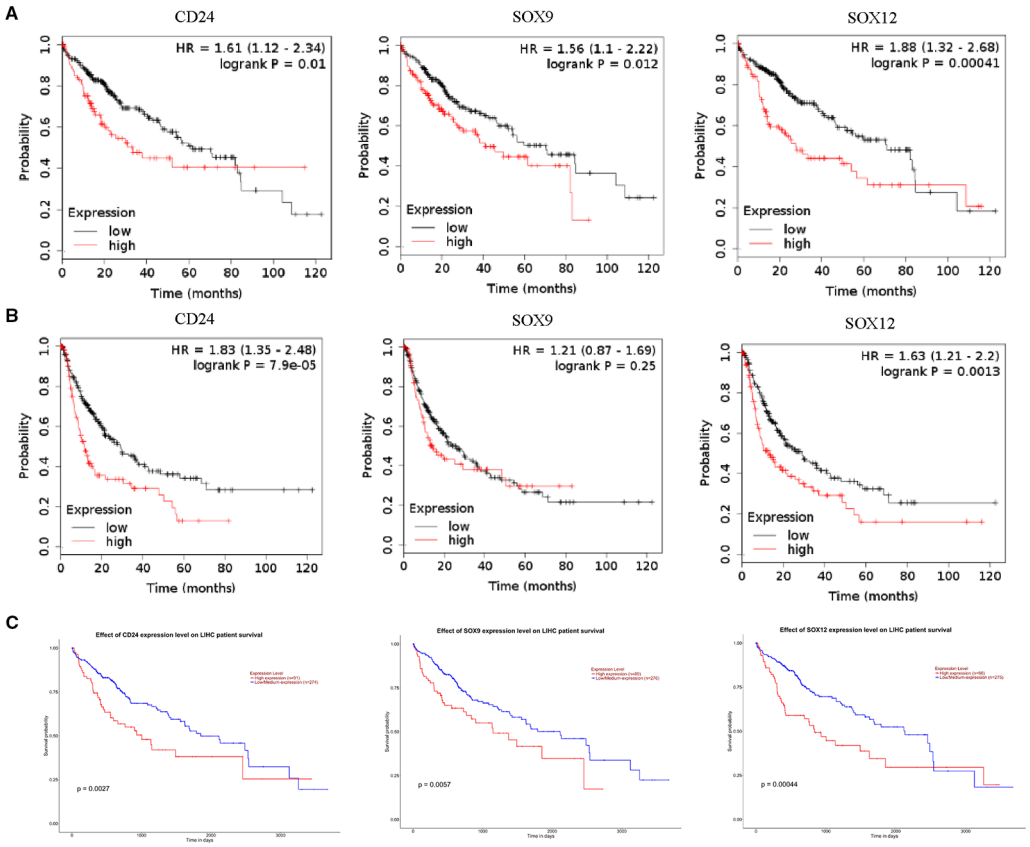

FIGURE 2 | Association of HCSC markers expression levels and prognosis of HCC. **(A)** Correlation of HCSC markers high expression levels with OS of HCC, n = 364, **(B)** correlation of HCSC markers high expression levels with PFS of HCC, n = 370, red font means negative correlation, green font means positive correlation.

Relationship Between HCSC Markers Expression and Immune Infiltration Level in HCC

Furthermore, the correlation of HCSC markers expressions with immune infiltration level in HCC from TIMER was investigated. The results suggested that some HCSC markers were increased with immune cell infiltration levels in HCC, while others were decreased or had no relationship (**Supplementary Table 1**). Expressions of *CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9*, *CD44*, *CK19,* and *CD47* were positively related to immune infiltration level in HCC, negatively related to tumor purity. Infiltrating levels of macrophage had the most significantly positive correlation with the eight genes, including *CD90* (r = 0.27, *P* = 7.69E-07), *EpCAM* (r = 0.36, *P* = 9.51E-12), *CD133* (r = 0.41, *P* = 2.35E-15), *CD24* (r = 0.39, *P* = 6.62E-14), *SOX9* (r = 0.28, *P* = 1.93E-07), *CD44* (r = 0.31, *P* = 4.82E-09), *CK19* (r = 0.39, *P* = 1.04E-13), and *CD47* (r = 0.26, *P* = 9.34E-07) (**Figure 4A**). Moreover, the second significant correlation was shown with CD4+ T cells, including *CD90* (r = 0.30, *P* = 9.11E-09), *EpCAM* (r = 0.27, *P* = 3.80E-07), *CD133* (r = 0.32, *P* = 1.47E-09), *CD24* (r = 0.31, *P* = 3.75E-09), *SOX9* (r = 0.30, *P* = 1.05E-08), *CD44* (r = 0.25, *P* = 2.01E-06), *CK19* (r = 0.39, *P* = 1.04E-13), and *CD47* (r = 0.21, *P* = 1.23E-04) (**Figure 4B**). In addition, *CD90* and *CD47* also showed remarkable positive correlations with infiltrating levels of dendritic cells (*CD90* r = 0.31, *P* = 5.06E-09, *CD47* r = 0.30, *P* = 1.17E-08), and *CD44* showed remarkable positive correlations with infiltrating levels of dendritic cells (r = 0.36, *P* = 6.16E-12) and neutrophils (r = 0.32, *P* = 1.48E-09) (**Supplementary Table 1**). Besides, *SOX12* was positively related with B cells (r = 0.215, *P* = 6.00E-05), CD8+ cells (r = 0.125, *P* = 2.09E-02) and macrophages (r = 0.208, *P* = 1.09E-04) (**Supplementary Table 1**).

Expressions of *ABCG2*, *ALDH1A1,* and *ALDH3A1* were negatively related with immune infiltration level in HCC, and showed no relation with tumor purity. *CD13* and *CD34* had no significant relationship with immune infiltration level in HCC (**Supplementary Table 1**), and their high expressions were related with good outcomes (**Figure 2**). Those findings suggested that *CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9*, *CD44*, *CK19*, *CD47,* and *SOX12* played specific roles in regulating

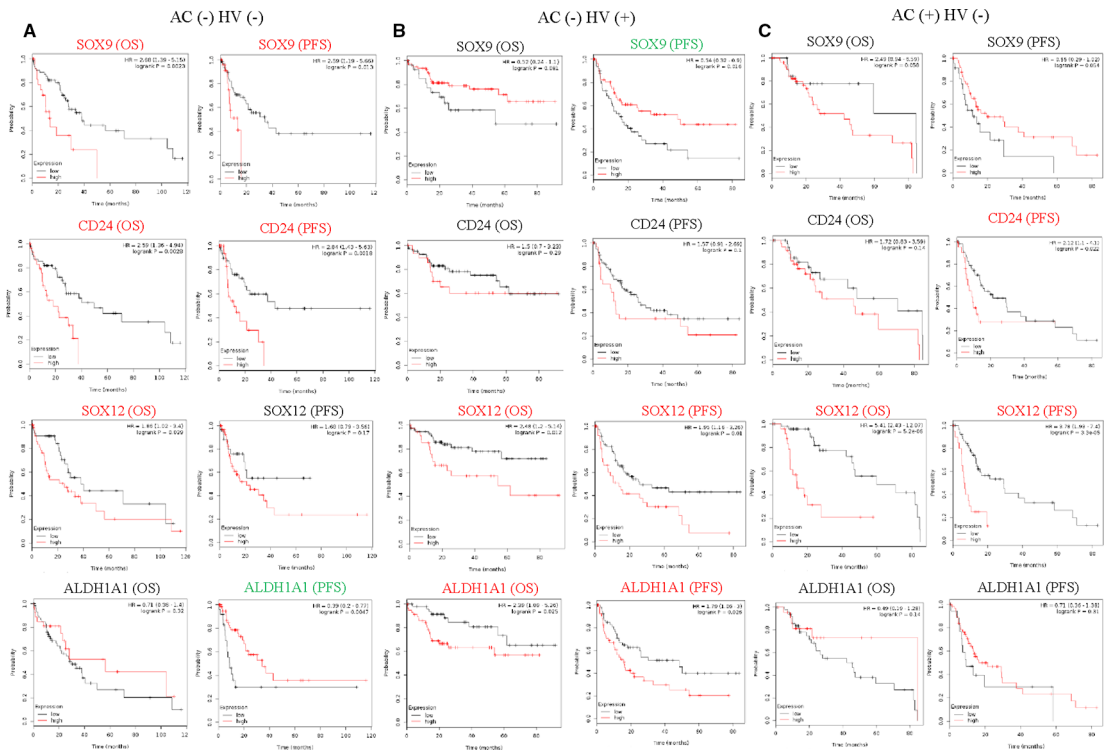

FIGURE 3 | Association of HCSC markers expression levels with prognosis of HCC with risk factors. **(A)** Correlation of HCSC markers high expression levels with OS (n = 91) and PFS (n = 91) of HCC without alcohol consumption (AC) and hepatitis virus (HV), **(B)** correlation of HCSC markers high expression levels with OS (n = 111) and PFS (n = 114) of HCC with HV, **(C)** correlation of HCSC markers high expression levels with OS (n = 76) and PFS (n = 78) of HCC with AC, red font means negative correlation, green font means positive correlation, black font means no correlation.

macrophage infiltration in HCC, which may play an important role in poor prognosis of HCC.

HCSC Markers Related Gene Regulatory Network in HCC

To better understand the immune influence of the 9 HCSC markers (*CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9 CD44*, *CK19*, *CD47*, and *SOX12*) expression in HCC, 4710 positively related genes with similar expression pattern with the nine HCSC markers were detected in HCC dataset of TCGA by GEPIA. Subsequently, the biological functions of the gene set were investigated by ClueGO and CluePedia analysis in Cytoscape software (**Figure 5A**). The majority biological function groups were involved in anatomical structure morphogenesis and development (**Figure 5B**, **Supplementary Table 2**). This was consistent with the properties of stem cells. Besides, GO items of innate immune response, adaptive immune response, humoral immune response, humoral immune response mediated by circulating immunoglobulin, immunoglobulin production and B cell mediated immunity, immunoglobulin mediated immune response were also significantly enriched in the network (**Figure 5C**, **Supplementary Table 2**). And there were 164 genes which took part in these immune GO items. Next, KEGG analysis of the 164 genes and 9 HCSC markers were conducted by DAVID. Hepatitis B and Hepatitis C pathway were significantly enriched, which were very closely related with HCC. Besides, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, RIG-I-like receptor signaling pathway and T cell receptor signaling pathway were also significantly enriched (**Supplementary Figure 7**, **Supplementary Table 3**). These findings suggested that the nine HCSC markers (*CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9*, *CD44*, *CK19*, *SOX12*, and *CD47*) were not only associated with immune infiltration, but also might impact the immune regulation.

DISCUSSION

Cancer stem cells (CSCs) have been identified in various human cancers (Sukowati, 2019). It was assumed that tumor growth is fueled by small numbers of tumor stem cell hidden in cancer, just as the renewal of healthy tissue (Clevers, 2011; Batlle and Clevers, 2017). Moreover, recent researches demonstrated that the CSCs are bound up with treatment resistance, tumor relapse and metastasis (Jordan et al., 2006). These findings may explain why tumor recurrence is the almost unavoidable outcome after radiation or chemotherapy. An increasing number of studies suggest that CSCs may be more profoundly impact on the cancer prognosis than we thought (Batlle and Clevers, 2017). Therefore,

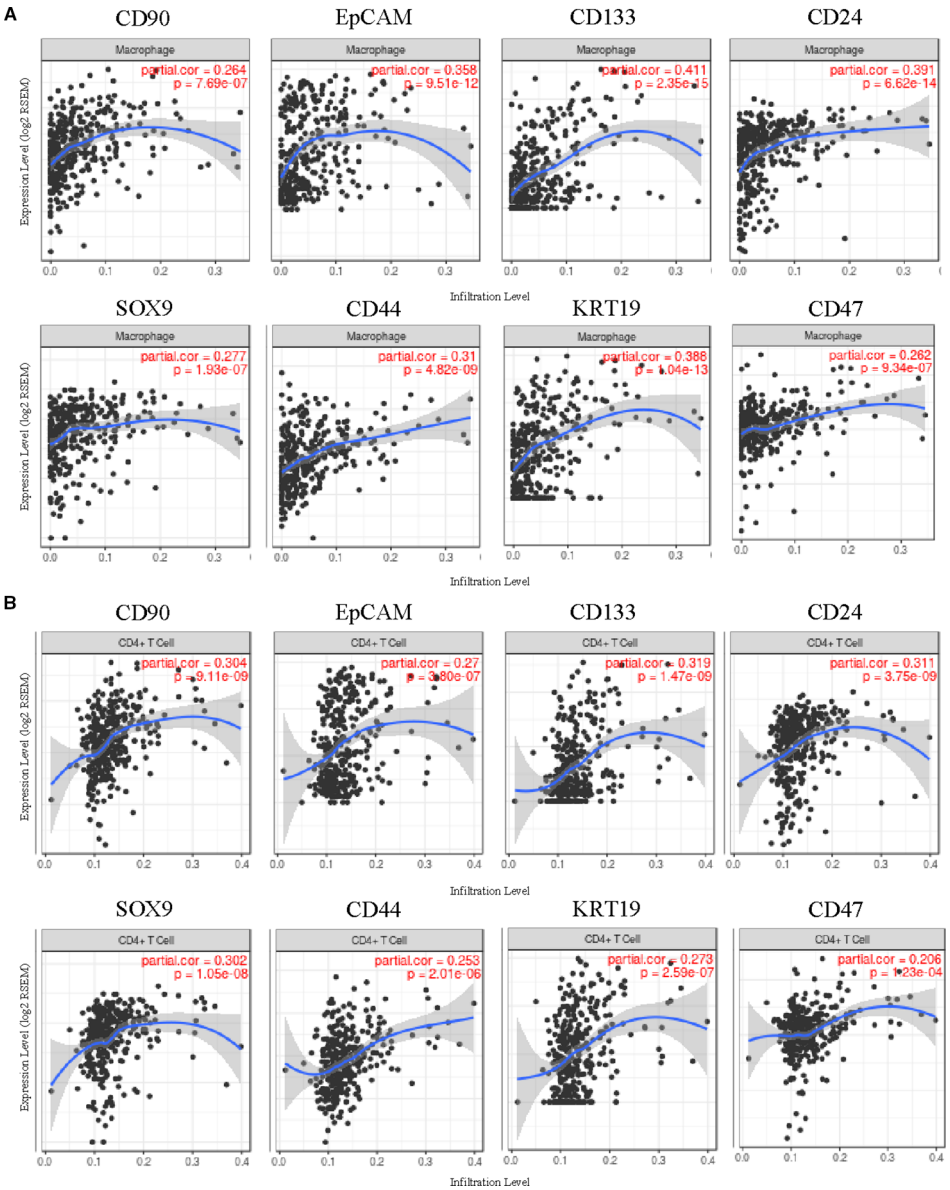

FIGURE 4 | Correlation of seven HCSC markers expression with immune infiltration level in HCC.

finding therapeutic targets on CSCs could be a more effective way for cancer treatment, including HCC. HCSCs are hierarchical cell populations of HCC, which are able to initiate and maintain tumor growth, and they have the dual properties of normal stem cells and tumor cells (Sukowati, 2019). As far as we know, CD90, EpCAM, CD133, CD24, CD13, CD34, SOX9, ABCG2, CD44, ALDH, CK19, SOX12, and CD47 are widely recognized as HCSC markers (Ma et al., 2008; Yang et al., 2008; Yamashita et al., 2009; Haraguchi et al., 2010; Lee et al., 2011; Zhang et al., 2013; Fernando et al., 2015; Kawai et al., 2015; Park et al., 2015; Li W. et al., 2017; Richtig et al., 2017; Zou et al., 2017; Rodríguez et al., 2018; Wang et al., 2019), whose combination may result in a wide variety of HCSC phenotypes. Up to date, the majority of HCSC studies focus on identification of the markers for the enriched cell populations that have high tumor initiation ability in immune-deficient mice. In the field of clinical research of human subjects, there is almost no report describing prognosis values of different HCSC markers, which may conform the cell line and animal experiment. In addition, few reports focus on the relationship between HCSC markers and immune infiltration in

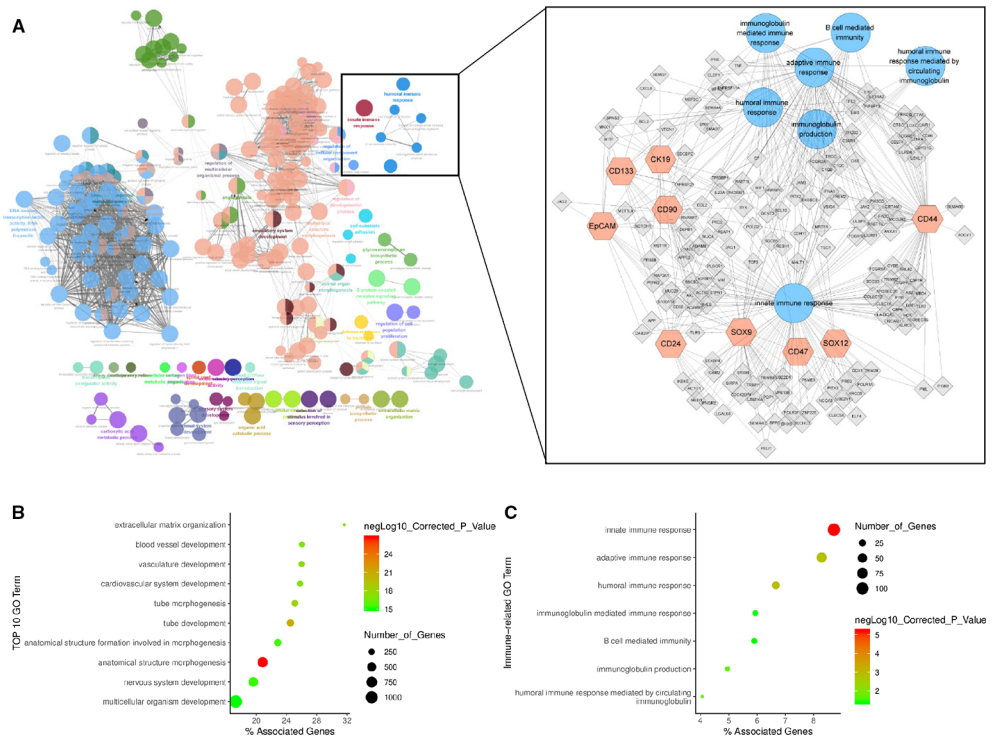

FIGURE 5 | HCSC markers related gene regulatory network. **(A)** HCSC markers related gene regulatory network. The network graph on the left shows all enriched GO items, with each dot representing a GO item. The network graph on the right is a detailed version of the immune-related GO items in the diagram on the left, with each dot representing a GO item, hexagon representing a HCSC mark gene, quadrilateral representing related gene, and line representing a correlation. **(B)** GO enrichment plot of biological function. **(C)** GO enrichment plot of immune related biological function.

HCC. Here, we for the first time reported the expression level of 14 HCSC markers which correlate to the prognosis of HCC under different conditions. Interestingly, we find that increased SOX12 expression can impact the prognosis of male HCC patients, and patients with viral infection and alcohol intake. Furthermore, our analyses show that in HCC immune infiltration levels are correlated with nine HCSC markers. Thus, our study provides insights in understanding the potential role of HCSC markers in tumor immunology.

In this study, we evaluated the mRNA expression level of the 14 HCSC markers in HCC by ONCOMINE, GEPIA, and HCCDB online database. The mRNA expression level of 10 HCSC markers was up-regulated in HCC in at least one database, including CD90, EpCAM, CD133, CD24, CD34, SOX9, ALDH1A1, ALDH3A1, CK19, and SOX12 (**Figures 1A–C**). Next, the expression of HCSC markers with tumor major stages of HCC was analyzed by GEPIA and UALCAN database. The expression level of *CD24* and *SOX12* in stage III was higher than that in stage I (**Figure 1E**, **Supplementary Figure 2B**), indicating that *CD24* and *SOX12* may have a role in terminal stage of HCC. Besides, high level of *SOX12* was significantly associated with axillary lymph nodes metastasis (**Supplementary Figure 2**). Previous study of immunohistochemistry staining for CD24 on human HCC tissue samples as well as their non-tumor counterparts showed there were 0% to 16% in the HCC specimens, whereas there was no CD24 expression in the non-tumor counterparts (Lee et al., 2011). It has also been proved that mRNA expression of SOX12 was dramatically upregulated in HCC tissues than in adjacent non-tumorous tissues. And mRNA expression of SOX12 was much higher in primary HCC tissues from patients who developed metastasis than that from those without metastasis (Huang et al., 2015).

Next, the influence of HCSC markers expression on prognosis of HCC was analyzed by Kaplan-Meier Plotter database. High expression of *CD24*, *SOX9,* and *SOX12* was negatively correlated with prognosis. In contrary, high expression of *CD13*, *CD34,* and *ALDH1A1* was positively correlated with prognosis (**Figure 2**). We also analyzed the correlation of HCSC markers expression with OS and PFS of HCC in different tumor stages. High expression of *CD24* and *SOX12* were both correlated with poor prognosis in stage I to IV, and *SOX9* was only correlated with poor prognosis in stage III and IV (**Supplementary Figures 4** and **5**). Immunohistochemistry of 166 HCC surgical specimens showed that compared to $SOX9^{-}$ patients, $SOX9^{+}$ patients had significantly poorer recurrence-free survival, stronger venous

invasion (Kawai et al., 2016). Our results on CD24, SOX9, and SOX12 are consistent with previous studies (Lee et al., 2011; Huang et al., 2015; Kawai et al., 2016). So far, most studies have not focused on mRNA expression of HCSC markers in different tumor stages. Our results indicated the significant distinction of tumor stages for certain HCSC markers expression. These findings emphasized a noticeable role of CD24, SOX9, and SOX12 in carcinogenesis and tumor progression in HCC. What we didn't expect was that many HCSC markers with high expression are negatively correlated with poor prognosis, such as *EpCAM*, *CD133,* and *CD13* in stage I to IV, *CD34,* and *CK19* in stage I and II, *ABCG2*, *ALDH1A1*, and *CD44* in stage III and IV (**Supplementary Figures 4** and **5**). As we know, liver has the ability of regeneration, and most of these markers are expressed in human liver multipotent progenitor cells (Dan et al., 2006; Kamiya and Inagaki, 2015). This suggests that HCSC markers may have duo functions for carcinogenic and regenerative mechanisms. Single marker may have limited effect on the poor prognosis of HCC. Hence, it is necessary to test HCSC markers in enough amount of cases to reveal the heterogeneity among cancer patients. At the same time, we have to bear in mind that these markers are also related with normal hepatic stem cell, which can facilitate tissue regeneration (Salama et al., 2010; Yoon et al., 2011; Rahman et al., 2014). Besides, in our results, high expression of *CD90*, *EpCAM*, *CD133*, or *CD44* was not significantly correlate with prognosis in HCC, while it was not the same in other papers (Zhao et al., 2016; Hu et al., 2018; Wendum et al., 2018). This indicated the complexity of HCSCs markers and more researches should be performed.

Hepatitis virus is the main risk factor for HCC (El-Serag, 2012). Hepatitis B and C, the carcinogenic viruses, may lead to HCC by inducing chronic inflammation (Read and Douglas, 2014). In our result, high expression of *SOX12*, *ALDH1A1,* and *ALDH3A1* is associated with poor HCC prognosis in the patients with hepatitis virus (**Figure 3B**). ALDH1A1 and ALDH3A1 are isotypes of ALDH gene family. Aldehyde dehydrogenase, which catalyze the oxidation of aldehydes to their corresponding carboxylic acids, play a major role in alcohol metabolism. Nonetheless, the activity of alcohol dehydrogenase in non-alcoholic fatty liver disease can also be increased (Jelski et al., 2018b). And previous studies have demonstrated the strong interactions between hepatitis virus and alcohol (McCartney et al., 2008). Due to the release of these enzymes from damaged liver cells, the ALDH activity was significantly higher in the sera of patients with hepatitis C than that in healthy persons (Jelski et al., 2018a). These evidences are consistent with our observation on the high expression of *ALDH1A1* and *ALDH3A1* in viral-infected liver cancer with poor prognosis. However, the mechanistic relationship between SOX12 and viral-infected liver cancer need to be further explored.

Previous studies have suggested that alcohol can directly initiate and promote liver cancer development and is associated with tumor progression (Chuang et al., 2015). In our study, high expression of *SOX12* was significantly related with poor prognosis of HCC patients who had alcohol consumption (**Figure 3C**). Not only that, we also found that SOX12 showed a close correlation with poor prognosis of male HCC patient, but no of female (**Supplementary Figures 3A, B**). As we know, gender disparities remarkably influence on the incidence and cumulative risk of liver cancer (Forner et al., 2018). Although previous studies have shown that overexpression of SOX12 promotes HCC metastasis and relates to poor prognosis (Huang et al., 2015; Jiang et al., 2017), there have been no reports about the significant difference of SOX12 in prognosis of HCC patients with different genders or alcohol consumption. As men consistently exceeded women in drinking frequencies and quantities (Wilsnack et al., 2000), the relationship among SOX12, gender and alcohol consumption is obscure, which needs to be further studied. In addition, how the virus or alcohol, gender and other risk factors aggravate the progress of liver cancer through SOX12 also needs our attention in the future. In this respect, virus- and alcohol-related interaction may be involved in the potential carcinogenic mechanism of HCSCs. Immunity plays an important role in the development of cancer and is the part of the adverse effects of both virus and alcohol. Thus, another important aspect of this study is that we investigated the correlation of HCSC markers expressions with immune infiltration level in HCC. Expressions of *CD90, EpCAM, CD133, CD24, SOX9, CD44*, *CK19*, *SOX12,* and *CD47* were positively related with immune infiltration level in HCC, especially with macrophages, and secondly with dendritic cells and neutrophils (**Figure 4**). Most of these genes are correlated with poor prognosis of HCC as analyzed before, implying the level of immune infiltration might be associated with HCSC markers' effect on poor clinical outcomes. The intrahepatic chronic inflammation microenvironment is currently perceived as a factor that facilitates the development of HCC and closely related to clinical prognosis (Galun, 2016), since TAMs produce factors that maintain cancer-related inflammation and potentiate tumor progression (Schoppmann et al., 2002). To further explore the mechanism of HCSCs, we constructed a HCSC markers related gene network, and performed GO and KEGG analysis. The pathway of leukocyte transendothelial migration explained infiltration of macrophages in HCC. Therefore, TAMs-related immune interaction could be a potential mechanism for HCSC markers.

In conclusion, our results suggest that seven upregulated HCSC markers (*CD90*, *EpCAM*, *CD133*, *CD24*, *SOX9, CK19,* and *SOX12*) are related with poor prognosis and immune infiltration in HCC. In addition, we find that high *SOX12* expression remarkably effect prognosis in male HCC patients but not in female. And HCC patients under viral infection or alcohol intake with increased *SOX12* expression had poorer prognosis. Therefore, HCSCs markers likely play an important role in tumor related immune infiltration and *SOX12* might be a potential therapeutic target in patients with HCC.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.oncomine.org/resource/login.html, http://gepia.cancer-pku.cn/index.html, http://lifeome.net/

database/hccdb, http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq, http://ualcan.path.uab.edu/index.html, https://cistrome.shinyapps.io/timer/, http://www.geneontology.org/.

AUTHOR CONTRIBUTIONS

Study concept and design: AX and FW. Acquisition of data: FW, XS, DW, and JL. Analysis and interpretation of data: AX, FW, XS, and DW. Statistical analysis: FW, XS, NZ, and XC. Drafting of the manuscript: FW, XS, DW, and SL. Critical revision and final approval of the manuscript: AX. Obtained funding: AX. Administrative, technical support: FW. Study supervision: AX and FW.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00112/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | 14 HCSC markers expression levels in HCC. **(A)** HCSC markers in data sets of HCC compared with normal tissues in Oncomine database. Cell color is determined by the best gene rank percentile for the analyses within the cell. **(B)** HCSC markers in data sets of HCC compared with normal tissues in GEPIA. Asterisk: $P < 0.01$. **(C)** HCSC markers in data sets of HCC compared with normal tissues in HCCDB.

SUPPLEMENTARY FIGURE 2 | **(A)** HCSC markers expression levels at tumor major stages in HCC in GEPIA. **(B)** Expression of CD24 in LIHC based on individual cancer stages. *P* value (Normal V.S Stage1) = 4.61E-12, *P* value (Normal V.S Stage2) = 6.32E-08, *P* value (Normal V.S Stage3) = 3.87E-09, *P* value (Stage1 V.S Stage3) = 0.0015. Expression of SOX12 in LIHC based on individual cancer stages. *P* value (Normal V.S Stage1) = 1.62E-12, *P* value (Normal V.S Stage2) = 3.99E-11, *P* value (Normal V.S Stage3) = 1.28E-09, *P* value (Normal V.S Stage4) = 0.0061, *P* value (Stage1 V.S Stage2) = 0.0427, *P* value (Stage1 V.S Stage3) = 0.0028. **(C)** Expression of CD24 in LIHC based on nodal metastasis status. *P* value (Normal V.S N0) = <1E-12, *P* value (Normal V.S N1) = 0.0064, *P* value (N0 V.S N1) = 0.0121. Expression of SOX12 in LIHC based on nodal metastasis status. *P* value (Normal V.S N0) = 1.62E-12, *P* value (Normal V.S N1) = 0.0433, *P* value (N0 V.S N1) = 0.0121. N0, no regional lymph node metastasis. N1, metastases in 1 to 3 axillary lymph nodes. TPM, Transcript per million.

SUPPLEMENTARY FIGURE 3 | Association of 14 HCSC markers expression levels and prognosis of HCC. **(A)** Correlation of HCSC markers high expression levels with OS of HCC, n=364, **(B)** correlation of HCSC markers high expression levels with PFS of HCC, n=370, red font means negative correlation, green font means positive correlation, black font means no correlation.

SUPPLEMENTARY FIGURE 4 | Association of HCSC markers expression levels and prognosis of HCC in stage I and II. **(A)** Correlation of HCSC markers high expression levels with OS of HCC in stage I and II, n=253, **(B)** correlation of HCSC markers high expression levels with PFS of HCC in stage I and II, n=256, red font means negative correlation, green font means positive correlation, black font means no correlation.

SUPPLEMENTARY FIGURE 5 | Association of HCSC markers expression levels and prognosis of HCC in stage III and IV. **(A)** Correlation of HCSC markers high expression levels with OS of HCC in stage III and IV, n=87, **(B)** correlation of HCSC markers high expression levels with PFS of HCC in stage III and IV, n=90, red font means negative correlation, green font means positive correlation, black font means no correlation.

SUPPLEMENTARY FIGURE 6 | Association of HCSC markers expression levels with prognosis of HCC with different gender. **(A)** Correlation of HCSC markers high expression levels with OS (n=246) and PFS (n=246) of male patient with HCC, **(B)** correlation of HCSC markers high expression levels with OS (n=118) and PFS (n=120) of female patient with HCC, red font means negative correlation, black font means no correlation. **(C)** Expression of HCSC markers in LIHC based on patient's gender. The median TPM and *P* value list in **Table 2**.

SUPPLEMENTARY FIGURE 7 | KEGG enrichment plot of KEGG pathway by functional annotation clustering.

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MINI REVIEW
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Organoid Models of Glioblastoma to Study Brain Tumor Stem Cells

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Glioblastoma represents an aggressive form of brain cancer characterized by poor prognosis and a 5-year survival rate of only 3–7%. Despite remarkable advances in brain tumor research in the past decades, very little has changed for patients, due in part to the recurrent nature of the disease and to the lack of suitable models to perform genotype-phenotype association studies and personalized drug screening. *In vitro* culture of cancer cells derived from patient biopsies has been fundamental in understanding tumor biology and for testing the effect of various drugs. These cultures emphasize the role of *in vitro* cancer stem cells (CSCs), which fuel tumor growth and are thought to be the cause of relapse after treatment. However, it has become clear over the years that a 2D monolayer culture of these CSCs has certain disadvantages, including the lack of heterogeneous cell-cell and cell-environment interactions, which can now be partially overcome by the introduction of 3D organoid cultures. This is a novel and expanding field of research and in this review, I describe the emerging 3D models of glioblastoma. I also discuss their potential to advance our knowledge of tumor biology and CSC heterogeneity, while debating their current limitations.

Keywords: glioblastoma, brain tumors, organoids, 3D models, cancer stem cells, neural stem cells, neurogenesis

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INTRODUCTION

The idea that tumor initiation, progression and regrowth after treatment are sustained by a subpopulation of cancer cells, the glioblastoma stem cells (GSCs), has been crucial to our current understating of glioblastoma (GBM) biology (Swartling et al., 2015; Alcantara Llaguno et al., 2016; Azzarelli et al., 2018; Hakes and Brand, 2019; Lu et al., 2019). Glioblastoma is a highly aggressive brain tumor characterized by elevated intratumor heterogeneity, which could be potentially attributed to variations in GSC behavior and stochastic consequences of their hierarchical growth pattern. Recent studies provided evidence for a proliferative hierarchy in GBM, by using a combination of experimental approaches, such as quantitative lineage tracing, clonal size dependences, mutational signature analysis, and single cell RNA sequencing (Patel et al., 2014; Tirosh et al., 2016; Lan et al., 2017; Neftel et al., 2019). Not only do these works indicated that tumor expansion follows a hierarchical lineage progression, but they also demonstrated that tumor cell fate decisions are rooted in a developmental program of neurogenesis. As such, early tumorigenesis is not primarily driven by genetic evolution, although genetic variations could still modulate the patterns self-renewal and differentiation of tumor cells, especially during later stages of disease progression. Evidence in another brain tumor originating in the cerebellum also showed that targeting the stem cell at the apex of a conserved developmental hierarchy could block tumor regrowth (Vanner et al., 2014). Thus, the cancer stem cell (CSC) model applied to GBM

provided a framework to understand tumor heterogeneity, predict tumor evolution, and might contribute to the identification of novel therapeutic targets aimed at eliminating the GSC in order to eradicate the tumor.

The simultaneous presence of different stem, progenitor, and differentiated cells along the developmental hierarchy and the high degree of intra-tumor heterogeneity render *in vitro* modeling of GBM particularly challenging. GSCs have been isolated from primary tumor biopsies and have been show to recapitulate *in vivo* tumor heterogeneity when forced to differentiate in culture or upon xenotransplantation (Galli et al., 2004; Singh et al., 2004; Pollard et al., 2009; **Figure 1**). However, when GSCs are grown in adherent 2D monolayer cultures, they lack intrinsic heterogeneity and 3D relative spatial organization, and lose interactions with the diverse components of the tumor extracellular matrix and the microenvironment. Moreover, these cells scarcely predict treatment efficacy, as drugs that initially proved effective in the context of cultured cell lines did not result in clinical applications (Zanders et al., 2019).

Thus, more refined model systems that allow the recapitulation of complex cancer phenotypes and yet retain the amenability to perform detailed analysis are needed, especially in view of the need to provide more accurate predictions of the therapeutic potential of new treatments. Encouraged by promising results in other cancer fields (Boj et al., 2015; Van De Wetering et al., 2015; Sachs et al., 2018; Tuveson and Clevers, 2019), several laboratories have directed their efforts to generate organoid models of glioblastoma, which consist, by definition, of 3D structures in which different cell types self-organize to establish appropriate cell–cell contacts and to create a microenvironment (Huch and Koo, 2015). As such, GBM organoids could better mimic tumor complexity and heterogeneity in growth potential and treatment responsiveness. This review describes the existing organoid GBM models that have just started to be developed and compares them to other 3D models, such as neurospheres and 3D bioprinted GBMs. It also discusses their potential to advance our understanding of GBM biology and to predict clinical outcome, while also considering their current limitations.

THREE-DIMENSIONAL MODELS OF GLIOBLASTOMA

Tumorspheres and Glioblastoma Organoids From Primary Tissue

Glioblastoma stem cells can be isolated from primary tumors and can be grown in culture for an extended period of time (Ignatova et al., 2002; Hemmati et al., 2003; Galli et al., 2004; Singh et al., 2004; Tunici et al., 2004; Yuan et al., 2004; Fael Al-Mayhani et al., 2009; Pollard et al., 2009; Vukicevic et al., 2010; **Figure 1** and **Table 1**). *In vitro* expansion of GSC is sustained by growth factors like EGF (Epidermal Growth Factor) and FGF2 (Fibroblast Growth Factor), conditions that also expand neural stem cells, highlighting the close relationship between GSC and their normal counterpart (Conti et al., 2005; Pollard et al., 2006, 2009). GSC can be grown in 2D adherent culture or as 3D neurospheres: the latter can be considered the very first "3D model" of GBM, as cells maintain a certain degree of polarization and 3D spatial organization (Galli et al., 2004; Azari et al., 2011). However, neurospheres are characterized by a necrotic core and can thus be able to achieve a maximum size of around 300 μm, before needing disruption and replating to survive (Reynolds and Weiss, 1992; Svendsen et al., 1998; Reynolds and Rietze, 2005). In addition, cells in neurospheres have lost their interaction with components of the extracellular matrix, and thus hardly mimic *in vivo* GSC behavior (**Table1**).

In 2016, the lab of Jeremy Rich developed *in vitro* conditions to grow 3D organoids from human GBM cells and from GBM biopsies. When embedded in matrigel, finely minced GBM specimens grew up to 3–4 mm in 2 months and could be kept in culture for over a year (**Table 1**), even if their growth slows down after several months, probably due to the limited diffusion of nutrients as the organoids grow in size (Hubert et al., 2016; **Figure 2A**). An interesting feature of these GBM organoids is that they recapitulate the gradient of stem cell density in relation to hypoxic levels found *in vivo*. The authors reported a high number of Sox2+ stem cells at the periphery of the organoid, while the core was characterized by lower abundance of Sox2+ cells and increased levels of hypoxia. Sox2+ stem cells also exhibited different molecular properties when located at the core or at the periphery of the organoids (Hubert et al., 2016). Thus, multiple Sox2+ populations may co-exist in organoids, suggesting that the organoid microenvironment might be able to sustain the simultaneous growth of different CSCs and would allow the study of cellular hierarchies in tumors. While highly promising, this system would require further characterization and validation across several GBMs. Indeed, establishment rates have yet to be determined and may be very patient-specific. Moreover, this system suffers from a relatively low to medium throughput capability and the long time necessary to establish the cultures (1–2 months) (**Table 1**).

Recently, a novel and faster protocol (1–2 weeks) of 3D GBM organoid derivation that overcome such limitations has been reported by collaboration between Donald O'Rourke, Guo-li Ming, and Hongjun Song at the University of Pennsylvania (**Table 1** and **Figure 1**). Instead of dissociating tumor biopsies to fine pieces, the authors cut the biopsies into around 1 mm fragments and culture them on an orbital shaker without matrigel and in serum free conditions not supplemented with EGF and FGF2 (**Figure 2B**; Jacob et al., 2020). These conditions should avoid selection of specific cell populations, thus better preserving inter- and intra-tumoral heterogeneity. These glioblastoma organoids, called GBOs, also developed hypoxic gradient and were further propagated in culture by cutting them into smaller pieces to avoid inner core necrosis. Importantly, these GBOs were cryopreserved and able to recover and continue their growth upon thawing. This is an essential step to generate GBM biobanks for subsequent recovery and analysis and the authors have currently biobanked around 70 GBOs from 53 patients (sometimes including different tumor subregions). GBOs largely maintain genetic and molecular signatures of the parental tumors. However, most analyses have been done within the first

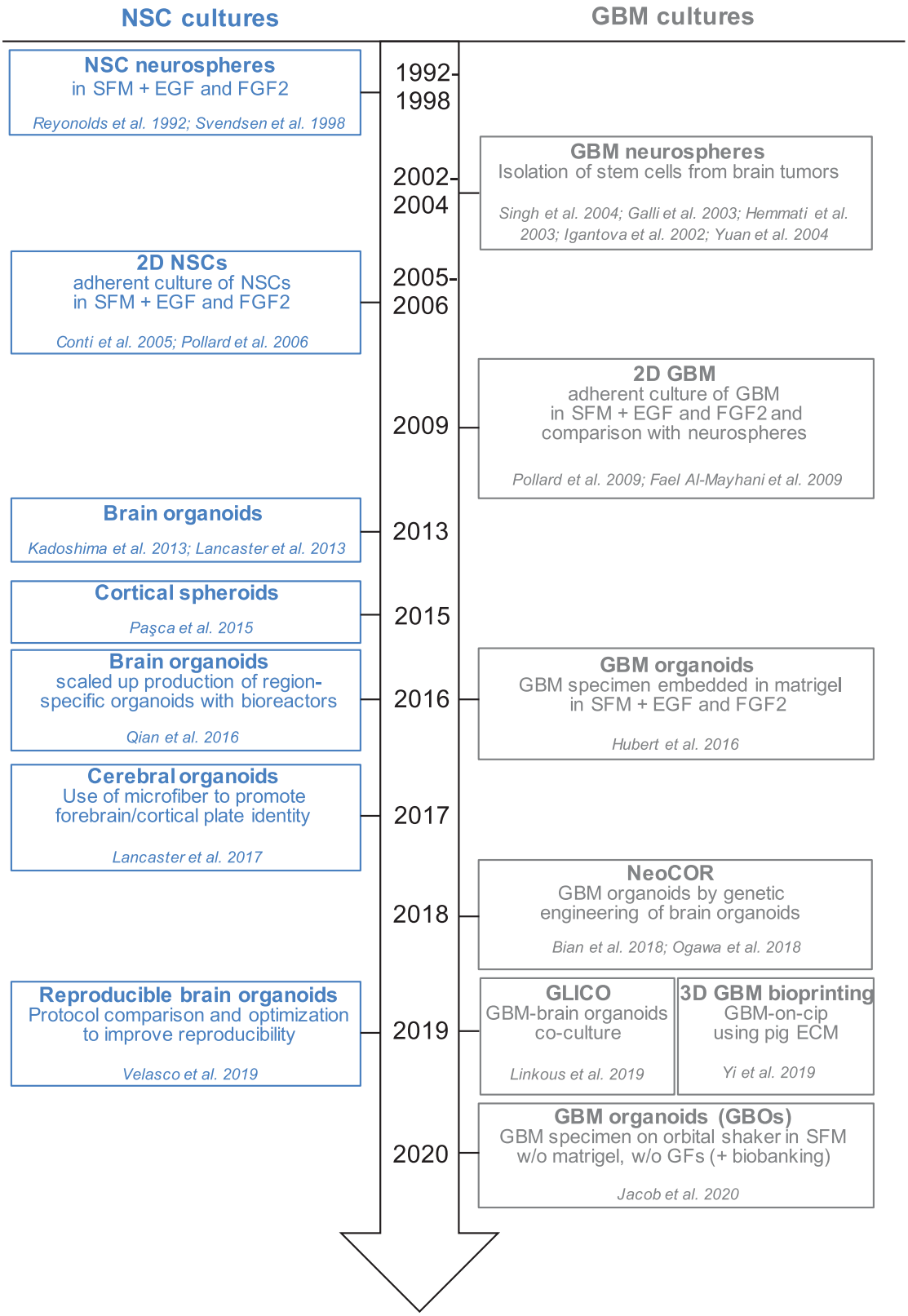

FIGURE 1 | Timeline of *in vitro* method to culture neural stem cells and glioblastoma cells in 2D and 3D. Schematic representation of the development of different protocols to culture NSCs (neural stem cells) and GBM (glioblastoma) cells in monolayer, spheres and organoids. SFM, serum free medium; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; GFs, growth factors; NeoCOR, neoplastic cerebral organoids; GLICO, GLioma cerebral organoids.

TABLE 1 | Overview of the characteristics of the different methods.

	2D	Spheres	Organoids			
			No matrigel GBO	Matrigel	Genetic Eng. NeoCOR	Co-culture GLICO
Efficiency of derivation	100%	<50%	91.4%	n.d.	Oncogene-dependent	100%
Time of culture establishment	1–2 weeks	1–2 weeks	1–2 weeks	1–2 months	1–4 months	1–2 months
Homogeneity (bulk analysis)	+	±	−	−	−	−
Heterogeneity (maintenance of tumor complexity)	−	−	+	+	+	+
Relative 3D spatial distribution	−	−	+	+	+	+
Genetic stability overtime	±[a]	±	+[b]	n.d[c]	n.a.[d]	+
Freeze/thaw	+	+	+	−	−	−
Maximum time in culture	>1 year	6–9 months	>1 month	>1 year	1–2 months post electroporation	14–24 days post co-culture
Potential to predict response to treatment	−	−	+	n.d.	+	+
GBM/non-GBM cell mix to study invasion	−	−	−	−	+	+
References	Fael Al-Mayhani et al., 2009; Pollard et al., 2009	Galli et al., 2004; Tunici et al., 2004; Pollard et al., 2009; Vukicevic et al., 2010	Jacob et al., 2020	Hubert et al., 2016	Bian et al., 2018; Ogawa et al., 2018	Ogawa et al., 2018; Linkous et al., 2019

[a]*Genomic stability is maintained within the first 6 months of culture (Pollard et al., 2009); some features are rapidly lost, such as EGFR amplification (Fael Al-Mayhani et al., 2009; Linkous et al., 2019).* [b]*Genomic stability is maintained, but the analysis has been done at only 2 weeks in culture (Jacob et al., 2020).* [c]*n.d. not determined.* [d]*n.a., not applicable. GBO, GBM organoid; NeoCOR, neoplastic cerebral organoids; GLICO, GLIoma cerebral organoids.*

two weeks in culture, as GBOs maintenance over very long periods has been variable.

This novel protocol is fast and reproducible and provides enough material for RNA and exome sequencing, as well as drug sensitivity tests. It is thus suitable for genotype-drug association studies and opens new avenues to personalized medicine approaches, along the line of current advances in other cancer fields in which organoid biobanks have already been established or are currently being generated (Boj et al., 2015; Van De Wetering et al., 2015; Sachs et al., 2018; Yan et al., 2018; Kim et al., 2019).

The establishment of the cultures within 1–2 weeks from surgical resection is particularly important, because current treatments are initiated within a month post-surgery and having preclinical information about potentially effective treatments might be extremely useful and might also help refine patient enrolment in clinical trials. In this direction, GBO treatment with CAR-T cells against EGFRvIII variant, which is currently in clinical trials (O'Rourke et al., 2017; Goff et al., 2019), resulted in specific effect only on GBOs containing high percentage of EGFRvIII+ cells, thus showing the translational impact and future pre-clinical potential of this approach (Jacob et al., 2020).

Tumor Development by Genetic Engineering of Brain Organoids

The development of human brain organoids or "minibrians" have revolutionized the way we operate in developmental neurobiology, by providing unprecedented access to aspects of human brain development functioning and disorders (Kadoshima et al., 2013; Lancaster et al., 2013, 2017; Paşca et al., 2015; Qian et al., 2016, 2018, 2019; Amin and Paşca, 2018; **Figure 1**). The potential of brain organoids to recapitulate aspects of brain cancer is particularly valuable as neither patient derived xenotransplantation in mice nor human brain tumor stem cells in 2D culture behave in the same way as tumors *in vivo*.

The labs of Jürgen Knoblich and Inder Verma have recently genetically engineered organoids to develop tumors (Bian et al., 2018; Ogawa et al., 2018; **Figures 1**, **2C** and **Table 1**). Bian et al. (2018) screened for genetic alterations that could lead to tumorigenesis and called the resulting tumor NeoCor (neoplastic cerebral organoids): the authors overexpressed known oncogenes

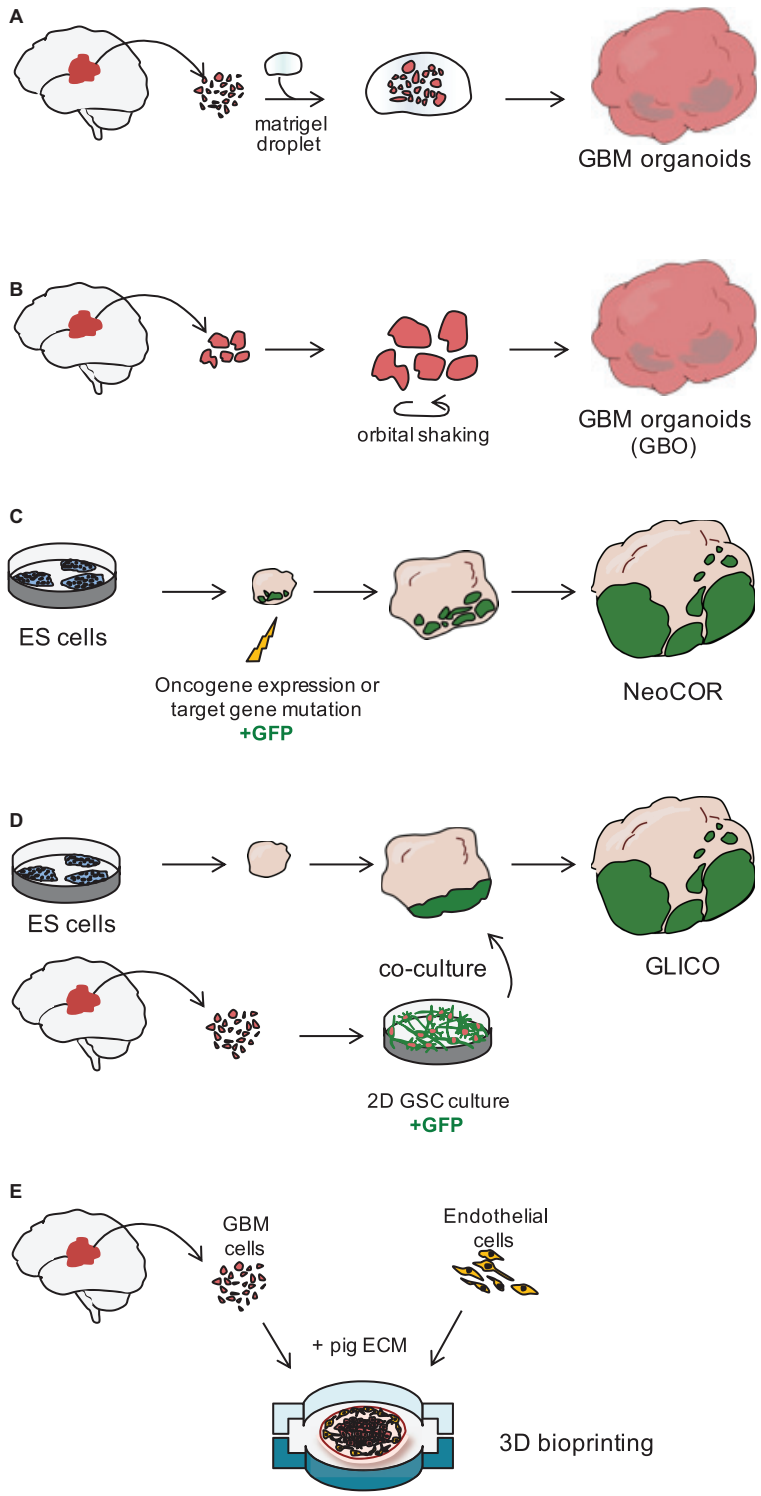

FIGURE 2 | Three dimensional models of glioblastoma. **(A)** Glioblastoma (GBM) organoids have been derived by embedding finely minced GBM specimen in matrigel (Hubert et al., 2016) or **(B)** by culturing pieces of tumor biopsies in defined matrigel-free and serum-free conditions, on an orbital shaker. GBO. GBM Organoid (Jacob et al., 2020). **(C)** Embryonic stem (ES) cell-derived brain organoids can be nucleofected at early stages of the differentiation to introduce tumor-promoting genetic alterations. During nucleofection, cells are also marked with green fluorescent protein (GFP) to visualize tumor cell growth. NeoCOR, neoplastic cerebral organoids (Bian et al., 2018; Ogawa et al., 2018). **(D)** Patient-derived glioblastoma stem cells (GSCs) are initially cultured in 2D, before being co-cultured with brain organoids. GSCs are marked by GFP to visualize integration and growth in the organoid. GLICO, GLIoma cerebral organoids (Ogawa et al., 2018; Linkous et al., 2019). **(E)** Patient-derived GBM cells and endothelial cells are seeded on a chip using a pig extracellular matrix (ECM) bio-ink and a 3D bioprinter (Yi et al., 2019).

by a transposase-based system and/or deleted tumor suppressor gene functions via CRIPSR Cas-9. Organoid cells have been targeted by nucleofection at very early stages of the differentiation and the cells carrying the genetic alterations have been marked by GFP, so that cell growth and tumor transformation could be easily followed. This screening identified MYC overexpression and a few more genetic combinations often found in human GBM to provide cells with a strong growth advantage. Transcriptomic profiling showed that MYC overexpressing tumors have a CNS-PNET-like identity (CNS-PNET: Primitive Neuro-Ectodermal Tumor of the Central Nervous System), while the other tumors resemble GBM more, suggesting that distinct genetic aberrations can induce tumors with specific cellular identities. Other works have also shown that mesenchymal GBM can be induced in organoids by defined genetic mutations, namely HRasG12V activation and p53 disruption (Ogawa et al., 2018).

Although these works show that a certain degree of GBM subtyping can be reproduced in organoids, whether all different GBM subtypes can be recapitulated in this system and how closely GBM-derived organoids resemble patient-derived GBM cells is still under investigated. Tumors other than GBM did not develop despite the genetic manipulation of genes classically altered in these tumors, such as the deletion of the inhibitory Sonic Hedgehog (SHH) receptor PTCH1 in SHH-group medulloblastoma (Bian et al., 2018). However, the oncogenic effect of PTCH1 deletion is known to be cell-type specific (Schüller et al., 2008) and might therefore necessitate organoid pre-differentiation down the cerebellar route for transformation to occur (Ballabio et al., 2020).

Co-culture of GBM Cells With Brain Organoids

Tumor models created by genetic engineering of organoids described above are particularly advantageous to effectively model GBM initiation, but they hardly recapitulate the genomic complexity of *in vivo* tumors, as the methodology requires genetic manipulation of the few known driver genes, which are not necessarily representative of the genomic GBM heterogeneity. The laboratory of Howard Fine and other laboratories have recently developed a novel approach that overcome this disadvantage, by co-culturing patient-derived GSCs with 3D brain organoids and called their model GLICO (GLIoma cerebral organoids; Linkous et al., 2019; **Figures 1**, **2D** and **Table 1**). The authors co-cultured different GFP-marked GSC cell lines with fully grown cerebral brain organoids and demonstrated that GSCs proliferate over time and integrate into the organoids. Each line behaves in a unique way, with some lines showing diffuse invasion, others forming "honeycomb"-like structures and others forming small regional nodes of proliferation (Linkous et al., 2019). Interestingly, co-cultured GSCs that exhibit higher degree of invasiveness were also more lethal when transplanted in mice (Ogawa et al., 2018). Thus, the observed heterogeneity in growth and invasion in the GLICO model likely reflects certain intrinsic properties of that particular patient-derived GSC line.

Cancer cells in this system not only displayed a cellular behavior that closely mimics the original tumor, but they also maintained key genetic aberrations of the native tumor. EGFR amplification, which was identified in two of the analyzed cell lines, was maintained in the GLICO models, while often lost in 2D cultures (Linkous et al., 2019; **Table 1**), indicating that this model may provide a more suitable microenvironment to preserve the genetic background of the *in vivo* tumor.

Three-Dimensional GBM Model via Bioprinting

The use of advanced 3D bioprinting technologies could enhance the way we design 3D GBM models *in vitro* (van Pel et al., 2018; Yi et al., 2019). Yi et al. (2019) created a GBM-on-chip model, in which they used decellularized pig brain extracellular matrix as a bio-ink to seed patient-derived cancer cells together with vascular endothelial cells in separated compartments (**Figure 2E**). Compartmentalization by seeding endothelial cells on the outside and cancer cells in the core of the chip established a radial oxygen gradient, which recapitulated important pathological features of the tumor. This model indeed exhibited hypoxia induced necrotic core, a perivascular niche and maintained a degree of spatial heterogeneity of the different cell types, with the higher number of Sox2+ stem cells at the periphery of the seeded tumor.

The "biomimetic" conditions of this system provided a microenvironment comparable to that of the original *in vivo* tumor tissue, offering the advantage of promoting cell-cell and cell-matrix interactions, and of better predicting treatment responses in a shorter time frame than other models (1–2 weeks compared to 1–2 months). However, the system still lacks accurate 3D spatial organization that can be only generated using self-assembled 3D organoid cultures, and it requires advanced technologies and expertise not always available to common biological laboratories.

ADVANTAGES AND LIMITATIONS: WHICH MODEL TO USE?

The best GBM model would be one that is complex enough to recapitulate features of the original tumor and simple enough to support investigation of different aspects of carcinogenesis in isolation. While the focus of this review is to look at emerging 3D *in vitro* models of GBM, several other approaches, including engineered mouse models or xenotransplantation, have been particularly useful to address tumor biology in other contexts [for a review of GBM models *in vivo* and *in vitro*, see Robertson et al. (2019)]. Thus, researchers might have to balance pros and cons of the different models to find the best fit for their research question, and might have to combine more than one model to take advantage of their complementary strengths (**Table 1**).

The development of 3D *in vitro* models of GBM holds great potential to study GBM biology and predict response to treatment, as they more closely recapitulate the complexity and heterogeneity of the original tumor. Indeed, most of the 3D organoid models described here have also shown selective vulnerabilities for targeted therapies or radiation that closely resemble tumor sensitivity *in vivo* (Hubert et al., 2016; Bian et al., 2018; Linkous et al., 2019). In addition, the recent establishment

of 3D GBM organoids from biopsies with a novel and faster protocol (1–2 weeks) promoted the generation of a live GBM biobank that can be used for genotype-drug association studies on a medium to high throughput capability (Jacob et al., 2020). Thus, 3D GBM models provide a powerful predicting tool that could be used one day to guide clinical decisions.

Some of the models described in this review (Bian et al., 2018; Ogawa et al., 2018; Linkous et al., 2019) and other similar models (da Silva et al., 2018; Plummer et al., 2019) allow the possibility to mix GBM and non-GBM brain cells (**Table 1**). This is particularly useful to study tumor invasion of the normal tissue and the interaction of tumor cells with normal brain cells. By targeting only one or the other compartment at a time, it will thus be possible to dissect the specific role of genes involved in cell-cell interaction, adhesion, guidance and migration, and this might identify novel therapeutical targets to block tumor infiltration.

The brain organoid tissue GBM cells interact with, however, resembles more an embryonic type of tissue, rather than the adult brain tissue of GBM derivation. At present, it is not clear how this might influence tumor properties. In the future, it would be interesting to develop a similar approach to model and study prenatal and childhood tumors, such as pediatric gliomas and medulloblastomas, which should maintain a closer link to their developmental origin (Liu and Zong, 2012; Azzarelli et al., 2018; Lu et al., 2019). As medulloblastoma did not develop in organoids, even when genetic alterations typical of this tumors were introduced, it might be necessary to generate regionalized organoids (Muguruma et al., 2015; Dias and Guillemot, 2017; Renner et al., 2017; Ballabio et al., 2020) tailored to the area of origin of that specific tumor, prior to transformation.

An aspect that has likely benefited from having this embryonic type of tissue, is the maintenance of the CSC compartment of the tumor. By providing cancer cells with the more appropriate environment that could support the simultaneous presence of different stem and progenitor cells, these 3D GBM organoid models will foster the investigation of CSC heterogeneity (Bhaduri et al., 2020). They will also open the possibility to study CSC developmental hierarchy in tumors and the influence of other cell types or of the environment on CSC fate decisions. While it is possible to incorporate non-neuronal cell types into organoids, such as microglia or other immune cells (Abud et al., 2017; Brownjohn et al., 2018; Ormel et al., 2018; Jacob et al., 2020), the main challenge still remains to recreate an environment that includes the vasculature and other cell types that could exhibit inflammatory and immunitary responses similar to an intact brain (Daviaud et al., 2018; Lancaster, 2018; Mansour et al., 2018).

The presence of different cell types and the high degree of heterogeneity is probably the main advantage and, at the same time, a disadvantage of the system because, while it reflects the complexity of the original tumor, it is probably the source of variability typical of these 3D cultures (Lancaster and Knoblich, 2014; Quadrato et al., 2017; Amin and Paşca, 2018; Qian et al., 2019; Velasco et al., 2019; **Table 1**). Thus, investigators might have to choose between growing cells in classical 2D monolayer or sphere cultures or in 3D organoids depending on whether they are more interested in performing bulk analysis on an homogenous cell population or whether they aim to investigate tumor aspects that requires maintenance of tumor complexity and heterogeneity.

CONCLUSION AND PERSPECTIVES

The emerging development of 3D organoids of GBM adds on to an abundance of choices to model this aggressive brain tumor (Robertson et al., 2019; **Figure 1** and **Table 1**). It provides researchers with an additional tool to understand GBM biology, and predict tumor progression and response to treatment. One of the main advantages of growing GBM in brain organoids is the possibility to simultaneously grow different stem cells, progenitors and their differentiated progeny within the same conditions (Hubert et al., 2016; Jacob et al., 2020). This not only mimics better the heterogeneity of the original tumor, but it also shows the persistence of developmental programs of neurogenesis in the tumor. By understanding how CSCs make fate decisions and by defining the aberrant developmental pathways that lead to tumorigenesis, it will be possible to exploit novel emerging vulnerabilities to kill or differentiate CSCs to eradicate the tumor.

Future challenges include the reduction of organoid variability, while maintaining tumor complexity and heterogeneity, and the incorporation of an appropriate microenvironment that could, for example, mimic inflammatory and immunological responses. This would be particularly relevant in view of current successes in cancer immunotherapy (Fesnak et al., 2016) and, once incorporated in organoids, it would help to understand how immunological response might influence CSC hierarchy, and tumor progression and regrowth (Weller et al., 2017; Lim et al., 2018).

Our knowledge on GBM has massively expanded in the past decades and future collaborations between oncologists, clinicians, and researchers in the cancer, stem cell, and developmental biology fields, together with the possibility to share different complementary models and tools are likely to bring the long-sought breakthroughs that will improve patient treatment and prognosis.

AUTHOR CONTRIBUTIONS

RA wrote the manuscript and prepared the figures.

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REVIEW
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Targeting Cancer Stem Cells by Genetically Engineered Chimeric Antigen Receptor T Cells

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The term cancer stem cell (CSC) starts 25 years ago with the evidence that CSC is a subpopulation of tumor cells that have renewal ability and can differentiate into several distinct linages. Therefore, CSCs play crucial role in the initiation and the maintenance of cancer. Moreover, it has been proposed throughout several studies that CSCs are behind the failure of the conventional chemo-/radiotherapy as well as cancer recurrence due to their ability to resist the therapy and their ability to re-regenerate. Thus, the need for targeted therapy to eliminate CSCs is crucial; for that reason, chimeric antigen receptor (CAR) T cells has currently been in use with high rate of success in leukemia and, to some degree, in patients with solid tumors. This review outlines the most common CSC populations and their common markers, in particular CD133, CD90, EpCAM, CD44, ALDH, and $EGFR^{VIII}$, the interaction between CSCs and the immune system, CAR T cell genetic engineering and signaling, CAR T cells in targeting CSCs, and the barriers in using CAR T cells as immunotherapy to treat solid cancers.

Keywords: cancer stem cell, chimeric antigen receptor T cell, chimeric antigen receptor T cell production, chimeric antigen receptor generations, chimeric antigen receptor T cell signaling

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INTRODUCTION

Cancer stem cells (CSCs) were initially identified in acute myeloid leukemia (AML) and subsequently in several solid tumors such as breast, brain, gastric, and prostate tumors (Lapidot et al., 1994; Bonnet and Dick, 1997; Al-Hajj et al., 2003; Hemmati et al., 2003; Collins et al., 2005; Fukuda et al., 2009; Gupta et al., 2009). Although CSC represents a subpopulation from the total tumor cells, it is the engine that supports cancer growth (Batlle and Clevers, 2017). Therefore, CSCs are major obstacles in tumor treatment because even with the high effectiveness seen with the current chemo-/radiotherapy to remove most of the cancer cells, cancer patients usually suffer from relapse and cancer recurrence due to CSCs resistance, renewal, and differentiation ability initiating new tumor in treated patients (Reya et al., 2001; Hong et al., 2015; Kaiser, 2015; Kaur G. et al., 2018). Thus, therapeutic approaches to eliminate CSCs are a necessity to overcome relapse and cancer recurrence in those patients.

Advances in immunotherapy and the development of chimeric antigen receptor (CAR) T cells have provided a solid and successful approach to target any protein expressed by cancer cells. CAR T cells' cytolytic capacity is independent of the major histocompatibility complex (MHC), and they are genetically engineered to express a target-specific antigen receptor (June and Sadelain, 2018). Clinically, a large number of patients with large B cell lymphoma (LBCL) and B cell acute

lymphoblastic leukemia (B-ALL) have shown total remission when treated with a single CAR T cell infusion (Maude et al., 2014b, 2018; Lee et al., 2015; Turtle et al., 2016a,b; Gardner et al., 2017; Neelapu et al., 2017; Fry et al., 2018; Park J.H. et al., 2018; Schuster et al., 2019). However, targeting solid tumors with CAR T cells was not associated with the same robust outcomes, but hope of success originates from some associated efficiency seen during early signs of clinical trials (Majzner and Mackall, 2019). Therefore, to use CAR T cells as a therapy to target CSCs, many efforts have been made to identify several markers to distinguish CSCs from other cancer cells (Codd et al., 2018). In the present review, CSC populations as well as their most common markers, the interaction between CSCs and the immune system, CAR T cells bioengineering and signaling pathways, clinical applications in targeting CSCs using immunotherapeutic approaches, in particular CAR T cells, and the barriers in using CAR T cells are discussed.

CSC POPULATIONS AND COMMON MARKERS

Tumor heterogeneity and development have been described in two models, the clonal evolution and CSC models (Marjanovic et al., 2013). The clonal evolution model proposes that stochastic events enable the selection and the advantageous growth of colonies that arose from the continuous acquisition of accumulated mutations. On the other hand, the CSC model suggests that particular tumor cells, which have the capacity to activate the expression of stem cell genes, are capable of driving tumor progression. These cells are thought to divide through asymmetric division, leading to the semipreservation of the parental cell genotype and the generation of a daughter cell that may pose novel mutations and not necessarily express stem cell genes. This controlled aspect of division is thought to enable the increase in heterogeneity in a hierarchical manner. CSCs may be rarer and less heterogeneous in early developed low-grade tumors (Alamir et al., 2018). In contrast, high-grade progressive tumors often have a highly varied heterogeneous population of CSCs, perhaps due to a weakened control on asymmetric cell division as more mutations are accumulated (Khan et al., 2018). Importantly, CSCs are tightly associated with the ability to initiate metastatic tumors and are inclined to be drug resistant (Aydemir Coban and Sahin, 2018). The CSCs model is gaining scientific popularity, as the clonal model is not always applicable to the formation of human cancers and does not sufficiently clarify the differences in the level of cancer heterogeneity between grades. Therefore, some have suggested dropping this model (Afify and Seno, 2019). However, for more details about clonal evolution, readers are referred to Marjanovic et al. (2013) and Afify and Seno (2019).

CSCs share many functional features with healthy stem cells including the ability to regenerate and proliferate extensively (Badrinath and Yoo, 2019). Although all types of CSCs identified until now have shared these properties as well as their resistance to the current therapy, each population identified in different tumor types such as breast, colon, brain, and leukemia has a unique marker and driver pathway (Desai et al., 2019). CSCs were identified 25 years ago in AML though transplanting the initiating AML cells into immunodeficient mice (SCID). These cells resided and proliferated in the bone marrow in response to cytokines treatment and generated leukemic cells similar in morphology to their counterpart in the original patients. Moreover, they found that these AML-initiating cells were $CD34^{+}CD38^{-}$ (Lapidot et al., 1994). Subsequently, several surface markers have been identified to distinguish leukemia stem cells (LSCs) including CD123, TIM3, CD47, CD96, CLL-1, and IL1RAP (Blair and Sutherland, 2000; Hosen et al., 2007; van Rhenen et al., 2007 Jin et al., 2009; Kikushige et al., 2010; Askmyr et al., 2013; Bruserud et al., 2017). The identification of these LSC surface markers has led to the generation of several promising therapeutic approaches targeting LSC of several hematopoietic malignancies, in particular those expressing CD123 (Busfield et al., 2014; Frankel et al., 2014; He et al., 2015; Chichili et al., 2015; Al-Hussaini et al., 2016; Ruella et al., 2016).

The first CSCs identified in solid tumor were of the breast tumor. These CSCs were characterized by the expression of CD44 and low levels of CD24 (Al-Hajj et al., 2003). Although several successful approaches have been reported in targeting brain CSCs (BCSCs), none of these therapies has been approved for targeting BCSCs (Desai et al., 2019). Ignatova et al. (2002) were the first to describe the brain CSCs. Since then, several characteristic markers for brain CSC have been documented including CD133 (Hemmati et al., 2003), CD49 (Lathia et al., 2010), L1CAM (Bao et al., 2012), and CD36 (Hale et al., 2014). Although the expression of these markers are different between patients and not sufficient on their own to designate brain CSC population, these markers are broadly used to identify adult brain CSCs (Desai et al., 2019). Moreover, CD133 and CD49 are expressed on both adult and pediatric brain CSCs regardless of the fact that both diseases are considered different. Therefore, targeting brain CSCs expressing CD133 in adults would provide a different outcome upon using the same approach with pediatrics (Desai et al., 2019). Colon CSCs share a phenotypic marker with the brain CSCs in which both were identified to express CD133 (Ricci-Vitiani et al., 2007), however, colon CSCs have been reported to express CD44 (Cheng et al., 2006), CD26 (Pang et al., 2010), as well as LGR5 (Schepers et al., 2012). Although preclinical trial targeting CD133-expressing cell has been a success (Ning et al., 2016), using combining therapies targeting both $LGR5^{+}$ colon CSCs and the differentiated tumor cells could show more success and prevent patient relapse (Shimokawa et al., 2017). CSC populations of other cancer types have also been described expressing different markers, and targeting these cells is considered as a promising therapy to treat the disease. The general features of the most commonly known markers to isolate solid cancer CSCs are discussed below.

CD133

CD133 is one of the most commonly used markers to identify CSCs of different tumors. CD133 is a product of a single-copy gene on chromosome 4 (4p15.33) in humans. The human gene consists of at least 37 exons spanning ∼160 kb. The transcript size is ∼4.4 kb. The transmembrane glycoprotein consists of 865

amino acids (aa) with a total molecular weight of 120 kDa. CD133 consists of five transmembrane glycoproteins. Despite that little is known about CD133 function, it has been reported to bind to cholesterol and found to be in the membrane protrusions (Visvader and Lindeman, 2008; Codd et al., 2018). Although CD133 has been accepted as a marker for CSCs, however, CD133 expression varies depending on the type of cancer, and it could be expressed on several noninitiating cancer cells as well as several healthy tissues and healthy stem cells (Shmelkov et al., 2008; Zhou et al., 2011). Therefore, CD133 cannot be used alone as a specific marker for CSCs. Moreover, using CD133 to detect CSCs has led to some inconsistent outcomes that might be due to their expression array and the detecting antibodies used (Hermansen et al., 2011). The antibodies to detect CD133 is usually mouse monoclonal antibodies against two different glycosylated epitopes, AC133 and AC141; therefore, the variation in the level of their glycosylation among the tissues could lead to false negative results (Codd et al., 2018).

CD90 and EpCAM

CD90 is a plasma membrane glycophosphatidylinositol anchor protein and is expressed in several tissues including skin and tissues of both the nervous as well as the olfactory systems (Sauzay et al., 2019). Recently, it has also been reported that CD90 is a marker expressed on the stem cells of the epidermis, liver, hematopoietic, and mesenchyme (Kumar et al., 2016). Moreover, several ligands for CD90 have been identified such as CD97, αv/β3, syndecan-4, CD90, and αx/β2 (Wandel et al., 2012; Kong et al., 2013; Leyton and Hagood, 2014). CD90 mainly function as an adhesion molecule, however, it is also involved in many other physiological functions including nerve regeneration and growth, migration as well as adhesion of leukocytes, apoptosis and activation of T cells, migration, and proliferation of the fibroblast (Rege and Hagood, 2006; Barker and Hagood, 2009; Bradley et al., 2009; Leyton and Hagood, 2014). Nowadays, CD90 is considered as a marker for CSCs in gastric and esophageal squamous cell carcinomas and hepatocellular carcinoma (HCC) due to the ability of tumor-isolated $CD90^{+}$ cells to generate cancer even upon the adoptive transfer of a very small number of these cells into immunodeficient mice compared to tumor-isolated $CD90^{-}$ cells (Yang et al., 2008; Jiang et al., 2012; Tang et al., 2013). Moreover, $CD90^{+}$ cells isolated from gliomas, lung, esophageal squamous cell carcinomas, and gastric cancers were able to regenerate and grow as a spheroid's *in vitro* serum free media (Kang and Kang, 2007; He et al., 2012; Jiang et al., 2012; Tang et al., 2013; Wang P. et al., 2013).

EpCAM is a transmembrane glycoprotein and is involved in cell adhesion as well as cells proliferation, differentiation, migration, signaling, and regeneration (Keller et al., 2019). Several studies have been using EpCAM plus CD44 as a marker for CSCs including CSC found in the liver, breast, prostate, colon, and pancreatic cancers (Yamashita et al., 2007; Gires et al., 2009).

CD44

CD44 is another common marker to identify CSCs in various cancer types, similar to CD133 and EpCAM. It is transmembrane glycoprotein, however, it has several functions such as a receptor for hyaluronic acid, as well as the ability to be involve in the adhesion, migration, proliferation. and survival of cells (Codd et al., 2018). Unfortunately, as with the abovementioned markers, CD44 is also expressed on healthy cells, making it difficult to be used to specifically differentiate CSCs. However, the ability of CD44 encoding gene to express multiple isoforms including CD44v, CD44s, and other variants gave the opportunity to identify that CD44v is highly expressed on tumor-capable cells compared to CD44s, while other variants have been identified to be associated with the progression of several cancer types (Mashita et al., 2014; Todaro et al., 2014; Thapa and Wilson, 2016). Furthermore, in head and neck cancer, it was found that tumor cells expressing high levels of CD44 are less immunogenic than $CD44^{lo}$ cells. The latter was associated to the PD-L1 high expression by $CD44^{hi}$ cells (Lee et al., 2016). Targeting CD44 binding domain by IgG1 antibodies during clinical trials showed high level of safety but modest effect in patients. This might be due to the crucial role that CD44 plays in T cells, in particular T helper (Th) 1 cells, in the proliferation, survival, memory function, and proinflammatory cytokines production (Baaten et al., 2010; Schumann et al., 2015; Menke-van der Houven van Oordt et al., 2016).

ALDH

Aldehyde dehydrogenase (ALDH) is a superfamily of 19 human isozymes and highly expressed in healthy as well as cancer cells with stem-like characteristics, however, ALDH expression is not limited to stem cells but also can be expressed by mature cells (Fillmore and Kuperwasser, 2008; Xu et al., 2015; Vassalli, 2019). ALDH is an enzyme that has the ability to oxide varied range of aldehydes, endogenous and exogenous, to their carboxylic acids to provide protection against oxidative stress. Moreover, ALDH have the ability to regulate cellular homeostasis through its role in the biosynthesis of the responsible molecules including retinoic acid (Marchitti et al., 2008; Jackson et al., 2011; Vassalli, 2019). ALDH roles have made it an attractive molecule in studying CSCs; therefore, many reports have identified ALDH as a specific marker for CSCs in several cancers. Moreover, healthy stem cells and CSCs can be differentiated by measuring the catalytic activity of ALDH that can also be used to monitor the prognosis of certain cancer patients (Ginestier et al., 2007; Deng et al., 2010; van den Hoogen et al., 2010; Marcato et al., 2011; Silva et al., 2011; Singh et al., 2015). With regard to ALDH association with stem cells, most of the focus has been placed on ALDH members that play role in the biosynthesis of retinoic acid via their cytosolic enzyme activity such as ALDH1 (Vassalli, 2019). ALDH1A1 is highly expressed by malignant CSCs in several cancers (Xu et al., 2015). Moreover, CSC uses ALDH to survive chemotherapy by blocking signal transducer and activator of transcription 3 (STAT3)–nuclear factor kappa B (NF-κB) signaling, a pathway that can diminish the accumulation of ALDH1A1 and sensitize tumor cells to chemotherapy (Canino et al., 2015; Zhao, 2016).

$EGFR^{VIII}$

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with a molecular mass ranging from 170 to

185 kDa (Weingaertner et al., 2013). Thirteen legends have been identified for EGFR activation such as epidermal growth factor (EGF); generally, activation via EGFR initiates several signaling pathways including Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K)/AKT, Janus kinase (JAK)/STAT, or phospholipase C (PLC)/protein kinase C (PKC) (Harris et al., 2003). Therefore, EGFR activation is involved in several cellular processes such as cell survival, proliferation, differentiation, apoptosis, and metabolism (Mendoza et al., 2011; van de Water et al., 2012; Jones and Rappoport, 2014; Treda et al., 2016). Several tumor-associated mutations of the EGFR gene have been identified. These include $EGFR^{VI}$ for the deletion of the N-terminal part, $EGFR^{VII}$ for the deletion of exons 14 and 15, $EGFR^{VIII}$ for the deletion of exons 2–7, $EGFR^{VIV}$ for the deletion of exons 25–27, and $EGFR^{VV}$ for the deletion of exons 5–28 (Wong et al., 1992; Cho et al., 2011; Guillaudeau et al., 2012; Francis et al., 2014). EGFR mutations are usually accompanied with prolonged signaling that is associated with metastasis, angiogenesis, apoptosis inhibition, and enhanced proliferation of the tumor cells (Nagane et al., 1996; Sangar et al., 2014). The EGFR amplification is associated with most of the glioblastoma (GBM) cases, with $EGFR^{VIII}$ being the most detected variant (Yamazaki et al., 1990; Wikstrand et al., 1995; Voldborg et al., 1997; Okamoto et al., 2003). In fact, EGFR gene is amplified in ∼50% of GBM patients, with 50–60% of the patients expressing $EGFR^{VIII}$. Moreover, $EGFR^{VIII}$ is rarely expressed in healthy tissues, making this exclusive tumor-mutated receptor an attractive therapeutic molecule (Wong et al., 1992; Moscatello et al., 1995; Del Vecchio and Wong, 2010; Snuderl et al., 2011; Del Vecchio et al., 2013).

Altogether, CSC markers have been shown to be useful for CSC enrichment. However, their utilization is limited due to the variability seen in their expression, which is perhaps caused by variation in the tumor microenvironment (TME). For instance, CD133 accuracy as a phenotypic marker for CSC is still controversial, in which several studies found that $CD133^{+}$ tissues are capable of regenerating tumor population with heterogenic properties *in vitro* and *in vivo*, whereas others reported that GBM cells expressing CD133 and $CD133^{-}$ cells have equal potential to generate tumor when transferred into nude mice (Singh et al., 2003, 2004; Beier et al., 2007). Moreover, it has been reported that some differentiated cancer cells have the ability to acquire stem-like characteristics displaying a great degree of phenotypic plasticity (Brooks et al., 2015). In breast cancer, two CSC subpopulations identified by $ALDH1^{+}$ and $CD44^{+}$ were found to have the potential to interconvert between themselves and with $ALDH1^{-}$ as well as $CD44^{-}$ nonCSCs (Liu S.L. et al., 2014). Therefore, it is crucial to understand the molecular foundations that regulate the expression of CSC markers and clarify their roles in maintaining CSC. Nevertheless, it is important to continue to uncover the nature of CSC markers, since their expression has been shown to correlate with patient survival in various types of solid cancers. Notably, CSC plasticity and heterogeneity are one of the challenging barriers that effect the patient's response to CAR T-cell therapy.

IMMUNITY AND CSCs

The components of the immune system play a complicated role in CSCs development. Macrophages are one of the most important cells of the innate immune system and can be polarized either into M1 or M2 macrophages (Ley, 2017). M1's main function is to defend the host by killing pathogens, virally infected cells, as well as cancer cells, while M2 clears the eliminated invaders by M1 and repairs the damage associated with the process of pathogen killing (Mills, 2012; Ley, 2017). M2 macrophages have also been reported to have mutual supportive relation with CSC development and growth. For instance, Jinushi et al. (2011) have reported that milk-fat globule EGF-8 (MFG-E8) producing M2 macrophages promote CSC resistance to anticancer drugs and tumorigenicity by activating their Sonic Hedgehog signals and Stat3 pathway. In addition to M2 macrophages' production of MFG-E8, M2 macrophages were also reported by Jinushi et al. (2011), to produce interleukin-6 (IL-6) that supports the same role as MFG-E8 in triggering CSCs' tumorigenicity and resistance to therapy. Moreover, it has been proposed that CSCs can enter latency stage and escape natural killer (NK) cells killing mechanism through downregulating the ligand that activate NK cells by expressing DKK1, a WNT pathway inhibitor (Malladi et al., 2016). It has also been reported that neutrophil extracellular trap released from activated neutrophils due to sustained lung inflammation can waken dormant tumor cells and initiate metastasis as well as cancer growth (Albrengues et al., 2018). These data support the notion of the importance of the interaction between CSCs and the immune system, however, since the reports are limited, more evidence are required to clarify and draw the whole picture of their interactions.

Generally, CSCs are immunosuppressive and can escape the immune system through several mechanisms to maintain their survival and establish resistant and heterogenic tumor (Prager et al., 2019). For instance, some CSCs escape the cytotoxic T cell killing process by downregulating their MHC class I (Di Tomaso et al., 2010; Schatton et al., 2010) or by decreasing their antigen processing capacity by reducing their low molecular weight protein and transporter associated with antigen processing (Di Tomaso et al., 2010). Furthermore, it has been reported that CSCs can partially mimic the expression of both their MHC class I and their inhibitory costimulatory molecules, such as PD-L1, with absences in the expression of their activating costimulatory molecules including CD80, CD86, and CD40. Upon contact with effector T cells, this improper stimulation induces effector T cells' anergy (Silver et al., 2016). This was supported by Parsa et al. (2007), in which they found that PD-L1 expressing tumor cells inhibited the activation and cytokine production by effector T cells via their direct interaction. An additional interesting mechanism was reported by Wei et al. (2010), in which they found that CSCs of GBM can induce naive as well as activated T cell apoptosis through galectin-3 secretion, allowing CSC expansion and depleting the intratumor effector cells of the immune system. It has also been reported that CSCs produce several anti-inflammatory cytokines including transforming growth factor beta (TGF-β) and IL-4 (Nappo et al., 2017; Prager et al., 2019). TGF-β is well known as an inducer

for both Tregs via FoxP3-independent and FoxP3-dependent pathways as well as pro-oncogenic M2 macrophages, to prevent effector T cell proliferation and to inactivate NK cells (Fantini et al., 2004; Thomas and Massague, 2005; Oh et al., 2017). M2 macrophages are induced by cancer cells and produce high levels of cytokines, express several enzymes including arginase 1 as well as protease and growth factors, all together promoting tumor growth and immunosuppression (Solinas et al., 2010; Weng et al., 2019). CSCs promote these cells' differentiation and recruitment from blood monocytes by producing periostin (Zhou et al., 2015) or direct interaction via CD90-CD11b and EphA4-Ephrin (Lu et al., 2014). Moreover, it has been reported that CSCs express inhibitory receptors such as CLTA-4 and PDL-1 on their surface to induce immunosuppressive cells. Although blocking those molecules has shown great success in clinical trials (Pardoll, 2012; Li S. et al., 2018), PDL-1 expression by CSCs is controversial, in which some studies reported PDL-1 expression on CSCs while others found it undetectable (Maccalli et al., 2014). Therefore, more studies are required to investigate other CSCs' immune evasion mechanisms to minimize tumor recurrence and metastasis. **Table 1** summarizes the various CSC identified mechanisms to modulate the immune system.

The immune system can eliminate CSCs either through antigen nonspecific mechanisms or through antigen-specific targeting-dependent approaches. NK cells are known for their ability to target and eliminate normal mesenchymal stem cells as well as various CSCs (Jewett et al., 2013; Ames et al., 2015a). This was seen in several studies targeting different types of CSCs, including GB, pancreatic, melanoma, oral, and lung CSCs; these studies documented that the main immune effector cells capable of targeting all these types of CSCs are the NK cells (Bui et al., 2015; Kozlowska et al., 2016, 2017). Moreover, NK cells are well known for their crucial role in killing cancer cells nonspecifically via recognizing the downregulation in the level of MHC class I (inhibitory signals) with the upregulation in the expression of the legends for NK-cell-activating receptors (activating signals) on the surface of the cancer cells. This equilibrium between NK cells activating and inhibitory signals is required for NK cell activation and effective antitumor killing function. Cancer cells are highly susceptible to NK cells killing, in particular, CSCs because they express lower levels of MHC class I than the rest of the tumor cells (Codd et al., 2018). However, some CSCs that are associated with certain cancer types can resist NK cell killing because they do not express NK-cell-activating legends (Wu et al., 2007; Wang et al., 2014). On the other hand, some CSCs express low levels of MHC class I as well as high levels of NK-cell-activating markers and therefore are more susceptible to killing by NK cells (Castriconi et al., 2009; Tseng et al., 2010; Tallerico et al., 2013).

CSCs can be identified from tumor-differentiated cells by MHC class I negative or decreased levels, CD54, PD-L1, as well as an increase in CD44 expression (Bui et al., 2015; Kozlowska et al., 2016). Jewett et al., have identified a maturational stage of NK cells in which the cells' CD16 expression levels are downregulated. NK cells at this stage of development were also characterized by their reduced cytotoxic ability upon interaction with CSCs, while interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) production is maintained, a functional state identified as "split anergy" (Jewett et al., 1997; Tseng et al., 2015a; Jewett et al., 2008). This functional state is reported to be essential for the tumor differentiation and potential NK cell inactivation (Bonavida et al., 1993; Jewett and Bonavida, 1996; Tseng et al., 2015a). Supernatants obtained from split anergy NK cells were

TABLE 1 | The various published mechanisms used by cancer stem cells (CSCs) to modulate the immune system responses.

Mechanisms by CSC to modulate the immune system responses		
1. Altering surface molecules expression		
Surface molecules	**Modulation**	**References**
a. MHC I, MHC II, and NKG2D ligand molecules	Decreasing MHC I and II without expressing NKG2D ligand molecules lower CSC immunogenicity and increase their immunosuppressive activities.	Di Tomaso et al., 2010
b. B7-H1 (PD-L1) and galectin-3	Increased expression of PD-L1 and secretion of galectin-3 by CSCs induces Tregs and inhibits the proliferation of effector T cells.	Wei et al., 2010
c. TLR-4	Reducing TLR-4 expression by CSCs elevates retinoblastoma-binding protein 5 that activates CSCs self-renewal ability.	Alvarado et al., 2017
d. MICA and MICB (ligands for stimulatory NK cell receptor: NKG2D).	Reducing MICA and MICB expression promote CSCs resistance to NK cytotoxic killing.	Wang et al., 2014
e. CD47	Overexpression of CD47 promotes CSC escape from bone marrow-derived macrophages phagocytosis.	Zhang et al., 2015
f. PD-L1	High expression of PD-L1by CSC induce T cell anergy and Tregs differentiation.	Hsu et al., 2018
g. CD133 and CXCR4	CD133 and CXCR4 expression by CSCs increase their tumorigenicity, metastasis and resistance to therapy.	Hermann et al., 2007
2. Secretion of anti-inflammatory molecules		
Secreted molecules	**Modulation**	**References**
a. Macrophages inhibitory cytokine 1 (MIC-1)	Production of MIC-1 by CSCs inhibit phagocytosis by macrophages and suppress T cell proliferation.	Wu et al., 2010
b. Macrophage migration inhibitory factor (MIF)	MIF secretion by CSC induces arginase 1 production from MDSC (myeloid-derived suppressor cell) that in turn inhibit antitumor T cell responses.	Otvos et al., 2016
c. IL-4	IL-4 production by CSCs enhances cancer growth, resistance to therapy and mediate effector T cells suppression.	Todaro et al., 2007; Volonte et al., 2014
d. TGF- β	TGF-β secretion by CSCs induces Tregs and M2 macrophages and prevent effector T cell proliferation and inactivate NK cells.	Fantini et al., 2004; Oh et al., 2017; Thomas and Massague, 2005

reported to mediate CSC differentiation mainly via IFN-γ and TNF-α, which in turn were documented to reduce the degree of tumor growth and induce tumor cell resistance to NK cell killing (Tseng et al., 2014, 2015a,b; Bui et al., 2015; Kaur K. et al., 2018). This was found to be associated with an increase in MHC class I, PD-L1, and CD54 expression and a reduction in CD44 levels on tumor cells. This was confirmed through adding anti-IFN-γ and anti-TNF-α antibodies to stimulated NK cells prior to their utilization in tumor differentiation; the antibodies inhibited the upregulation of these markers on the cancer cells (Tseng et al., 2014, 2015a,b). In addition, Ames and colleagues have reported that CSCs from various cell lines, as well as those isolated from primary tumor specimens based on the expression of several CSC markers including CD24, CD44, CD133, and ALDH, are eliminated preferentially by activated NK cells. This was dependent on the expression of several NK cell activation markers on CSCs including MICA/B, Fas, and Death receptor 5. Moreover, adoptive transfer studies have shown that the adoptive transfer of stimulated NK cells into orthotopic human pancreatic cancer tumor-bearing mice significantly reduced intratumoral CSCs as well as tumor burden (Ames et al., 2015a). The same group have also published that *ex vivo* stimulated NK cells are capable of targeting solid cancers CSCs *in vitro* postCSCs radiation, which was found to increase the number of CSCs expressing stress ligands such as MICA/B and Fas. Upon adoptive transfer along with radiotherapy, locally radiated tumor-bearing mice survival was prolonged (Ames et al., 2015b). Although CSCs are highly susceptible to NK cell killing, the report of Castriconi et al. (2009), shows that NK cells isolated from GBM patients are incapable of killing CSCs, despite that cytokines activated NK cells isolated from healthy donors were able to eliminate CSCs. These data points at the importance of the TME in NK cell function in killing CSCs, as well as their possible role in modulating CSC phenotype to evade NK cell's killing mechanisms.

TME plays a curtail role in NK-cell-mediated cytotoxicity and can prevent NK cell function via two major approaches: suppression of NK cells and evasion via immunoediting of the tumor cells. At the tumor site, the TME favors type 2 over type 1 responses that may suppress the infiltrated NK cells upon their interactions with tumor (Vitale et al., 2014). Tumor-associated cells residing at the tumor site, including immature dendritic cells (DCs), Tregs, tumor-associated macrophages, and myeloid-derived suppressor cells, produce various molecules such as TGF-β, IL-4, IL-10, prostaglandin E2, and idoleamine 2,3-dioxygenase (Stojanovic et al., 2013; Konjevic et al., 2019). These molecules enable the tumor to downregulate NK-cell-activating receptors including NKp30, NKp44, or NKG2D, as well as tumor necrosis factor-related apoptosis-inducing ligand (Baginska et al., 2013; Vitale et al., 2014; Zenarruzabeitia et al., 2017; Park A. et al., 2018; Nayyar et al., 2019; Konjevic et al., 2019). For instance, TGF-β can inhibit the expression of NK cell receptors including NKp30 and NKG2D, which is essential for tumor recognition and elimination by NK cells and for their productive interaction with DCs (Castriconi et al., 2003). Similarly, NK cells' potential to eliminate tumor cells and functional interaction with DCs can be reduced by IL-4 produced and released into the TME (Marcenaro et al., 2005). Besides molecule production by tumor residence cells, immune cells at the tumor site can modulate NK cell function by competing for IL-2 or inhibiting NK cell IL-2-mediated activation via cell-to-cell contact (Sitrin et al., 2013; Sprinzl et al., 2013). TME is often associated with hypoxia, which has been reported to significantly suppress both the expression and function of NK cells' major activating receptors (Balsamo et al., 2013). As mentioned earlier, tumor cells can evade NK cells via immunoediting, which can occur due to chronic exposure of tumor cells to NK cells. For example, tumor-resistant melanoma cells cocultured with NK cells displayed an increased level of MHC class I (Balsamo et al., 2012). Collectively, these mechanisms could disturb the equilibrium between NK cell activation and inhibitory signals. Several other TME factors are reported to modulate NK cell cytotoxic function including the TME influence on NK cell metabolism. However, NK cells are not the focus of this review; therefore, for full comprehensive discussion, readers are referred to Terren et al. (2019) and Chambers et al. (2018).

T-cell receptor (TCR) divides the T cells into two populations: αβ TCR and γδ TCR T cells. Unlike αβ T cells that are MHC-dependent, γδ T cell activation is direct and independent of MHC molecules (Sebestyen et al., 2019). The protective role of γδ T cells in cancer was first reported in a mouse model of cutaneous squamous cell carcinoma, in which the adoptive transfer of γδ T cells into mice deficient of γδ T cells prevent the cancer development (Girardi et al., 2001). Subsequently, several studies reported the key protective role that γδ T cells play in preventing cancer. γδ T cell protection against cancer is mainly reported to be through the production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-17 as well as through their cytotoxic ability (Ma et al., 2011; Sebestyen et al., 2019). However, clinical trials stimulating γδ T cells or even transferring γδ T cells with or without activating stimuli into cancer patients show very low efficiency and very limited success (Wilhelm et al., 2003; Dieli et al., 2007; Bennouna et al., 2010; Meraviglia et al., 2010; Nakajima et al., 2010; Lang et al., 2011; Kobayashi et al., 2011; Kunzmann et al., 2012; Bregeon et al., 2012; Wada et al., 2014; Pressey et al., 2016; Aoki et al., 2017). This might be due to the lack of knowledge regarding the specificity and diversity of these cells. γδ T cells are characterized by their ability to recognize early metabolic changes including stress-induced self-antigens that differentiate healthy cells from transforming one. Therefore, identifying the proper activating process of γδ T cells as well as their receptors would lead to successful identification of tumor cells with very low mutational changes at early stages, unlike any other immunotherapeutic approaches (Sebestyen et al., 2019). As mentioned earlier, the adoptive transfer of γδ T cells into cancer patients was not that successful but was associated with high level of safety; therefore, γδ T cells are currently suggested to be used as CAR carriers (Fisher and Anderson, 2018; Liu et al., 2019). Similar to antitumor CAR NK cells that have been reported to be associated with less harmful side effects, such as cytokine release syndrome (CRS), γδ T cells are postulated to be associated with the same level of safety (Li Y. et al., 2018). Nevertheless, γδ T cells and NK cells can eventually be educated due to their tight control by several receptors such as natural cytotoxicity

and killer-cell immunoglobulin-like receptors (Orr and Lanier, 2010). Additional probable issue with using γδ T cells as a CAR carrier cells is the possible long survival of these cells, as has been documented for the NK cells; moreover, the metabolic changes that γδ T cells recognize can occur in normal cells postexposure to stressful conditions (de Witte et al., 2018). Furthermore, using γδ T cells as a CAR carrier will not clear up the issue of identifying target independent of the changes load that γδ T cells recognize in transforming cells (Hartmann et al., 2017; Sebestyen et al., 2019).

CD8 T cells represent the major tumor killer cells of the adoptive immune system. Generally, cancer cells including CSCs express MHC class I but not MHC class II, and CD8 T cells recognize cancer antigens in a specific manner depending on the proper presentation of antigens on MHC class I as well as on the level of MHC class I (Codd et al., 2018). However, CSC targeting by CD8 T cells has been reported to be either resistant or susceptible to T cell killing depending on the type of cancer and origin and culture conditions of the cells (Codd et al., 2018). Several antigens have been documented to be specifically expressed on MHC class I of the CSCs such as cancer/testis (CT) antigens. CT antigens are expressed exclusively on germ cells but can reappear in some cancer cells (Codd et al., 2018). One example of CT antigens that have been found to be solely expressed on CSCs is the brother of the regulator of the imprinted site (BORIS), which is found to be expressed on CSCs of cervical as well as lung cancers, and can be targeted successfully by specific CD8 T cells (Asano et al., 2016; Horibe et al., 2017). CT antigens are classified as one of the tumor-associated antigen (TAA) family, however, for a full comprehensive review on TAA as well as CT antigens, the reader can refer to this reference Hirohashi et al. (2016).

CSC-SPECIFIC TARGETING BY CAR T CELLS IN CLINICAL APPLICATIONS

Several immunotherapeutic approaches to treat cancers have been developed including monoclonal antibodies, adoptive T cell therapy, immune checkpoint inhibitors, oncolytic virus therapy, and cancer vaccines. All of these therapies are still under extensive investigations and are associated with shared advantages as well as disadvantages. Immunotherapeutic medicine is characterized and differs from the traditional cancer therapy by being highly selective to tumor cells and is not associated with unpleasant side effects. Although immunotherapies are not free from adverse side effects, as these therapies are developing and evolving, the side effects become more controllable. Moreover, immunotherapies can stimulate the immune system against cancer for a long period and, therefore, might provide long-term remission and reduce tumor recurrence. However, the long-term influence and efficiencies are still unclear. As the immune system has the ability to eliminate almost all types of cancer cells, designing immunotherapy that allow immunity to perform such a function will be a very beneficial challenge to overcome. As with many treatments, immunotherapies are associated with some disadvantages, and one of the major obstacles is the high cost and the intensive labor required to produce the treatment. Immune checkpoint inhibitors are considered the most attractive treatment among all of the available immunotherapies due to the long-term benefits seen in melanoma, Hodgkin's lymphoma, and Merkel cell carcinoma patients (Schmidt, 2017). Nevertheless, similar to CAR T cell treatment and other clinically used immunotherapies, immune checkpoint inhibitors are not beneficial to all patients and the benefited patients can suffer from acquired resistance. Generally, acquired resistance includes loss of target antigens, particularly seen with T cell adoptive therapies, upregulation of the expression of immune checkpoint legends such as PD-L1 on target cells, and accumulation in Tregs at TME (Sharma et al., 2017; Thommen and Schumacher, 2018). Although CAR T cells are associated with several disadvantages such as their restricted efficiency, systemic immunogenicity, undesirable toxicity, and high cost as well as the extensive time that is required for production, the huge success seen in their use with hematological malignances and the continued investigations to overcome all these obstacles make CAR T cells a hugely promising therapy to treat cancers. Nevertheless, all immunotherapeutic approaches including immune checkpoint inhibitors are still at their initial steps of development and, therefore, are associated with challenges that have to be further studied and resolved, including Treg induction, toxicity, primary as well as acquired resistance, and limited efficiency.

Most of the reported clinical trials using immunotherapeutic approaches to target CSCs mainly rely on loading CSCs isolated from cancerous tissues into DCs and then transferring the DCs to the patients as a vaccine. The list of the available immunotherapy targeting CSCs can be found at http://clinicaltrials.gov, and more details can be found in the following reference (Wefers et al., 2018). As this review mainly focuses on CAR T cells in targeting CSCs, the following sections discussed CAR T cells in details.

Genetically Engineered CAR T Cells: Production, Generations, and Signaling

CAR consists of three domains: extracellular domain, which binds to the target antigens, transmembrane domain, and intracellular signaling domain (Kuwana et al., 1987; Gross et al., 1989; Finney et al., 1998; Maher et al., 2002; Sadelain et al., 2013). Engineering CAR T cells starts with the collection of autologous cells from the patient and, subsequently, T cell enrichment and pure isolation by various methods, including gradient density to isolate peripheral blood mononuclear cells (PBMCs) and magnetic-bead-labeled antibodies to purify T cells (Powell et al., 2009; Riddell et al., 2014; Levine et al., 2017). During T cell activation *in vitro* mainly with anti-CD3 and anti-CD28 antibodies-coated beads, the viral vector using murine retroviruses or lentiviruses is added to the activated T cells (Levine et al., 2017). The viral vectors to produce CAR T cells express the genes responsible for the viral infection pathway without the genes that are associated with the virus toxicity and replication (Thomas et al., 2003). To produce viral vector, the unwanted encoding regions for virus toxicity and replication in the virus genome are deleted, while the sequences that are needed for packaging the virus capsid from the vector genome

or required for the viral DNA integration are left intact in the virus genome (Thomas et al., 2003). The CAR genetic materials are then cloned into the viral genome replacing the deleted genes producing vector genome encoding CAR genetic information. A separate packaging constrict is used to aid in the replication of the modified viral genome in the packaging cells, in which the deleted genes encoding the viral replication as well as the viral capsid proteins are included in this constrict (Thomas et al., 2003). Subsequently, both the vector genome plus the packaging constrict are cotransfected into a packaging cell line and expressed as recombinant viral vector particles. The RNA of the produced recombinant viral vector is reverse transcribed into DNA, which in turn integrates the genome of the patient T cells permanently to maintain CAR expression as the cells proliferated and increased in numbers in a bioreactor (Levine et al., 2017). Subsequently, the integrated CAR DNA is then transcripted into messenger RNA (mRNA) and eventually translated into CAR expressed on the surface of the patient T cells (**Figure 1**). The optimal number of recombinant viral vectors to transduce and integrate the specific CAR sequence into the T cells, known as multiplicity of infection (MOI), always has to be optimized to obtain the highest expression level of CAR in T cells. It would require long-term monitoring to determine the level of safety of using viral vectors in CAR T cells, however, no reported adverse events to viral vectors have been documented so far (Cavazzana-Calvo et al., 2010; Aiuti et al., 2013; Biffi et al., 2013; McGarrity et al., 2013; Sessa et al., 2016). Notably, one of the CAR T cell therapy limitations is the persistence of the cells that might be due to the integration nature of the viral vector (Maude et al., 2014b). Moreover, patients that have received CAR T cells of viral-based vectors, namely lentiviral, might test positive for HIV. Therefore, several other approaches have been used to generate CAR T cells such as the Sleeping Beauty transposon system or mRNA transfection, however, engineering CAR T cells using viral vector, particularly lentivirus, as discussed above, is considered the most effective until now. **Table 2** illustrates the advantages and disadvantages associated with each CAR T-cell-producing approach.

The extracellular domain of CAR consists of a single-chain variable fragment (scFv), which is derived from the variable heavy

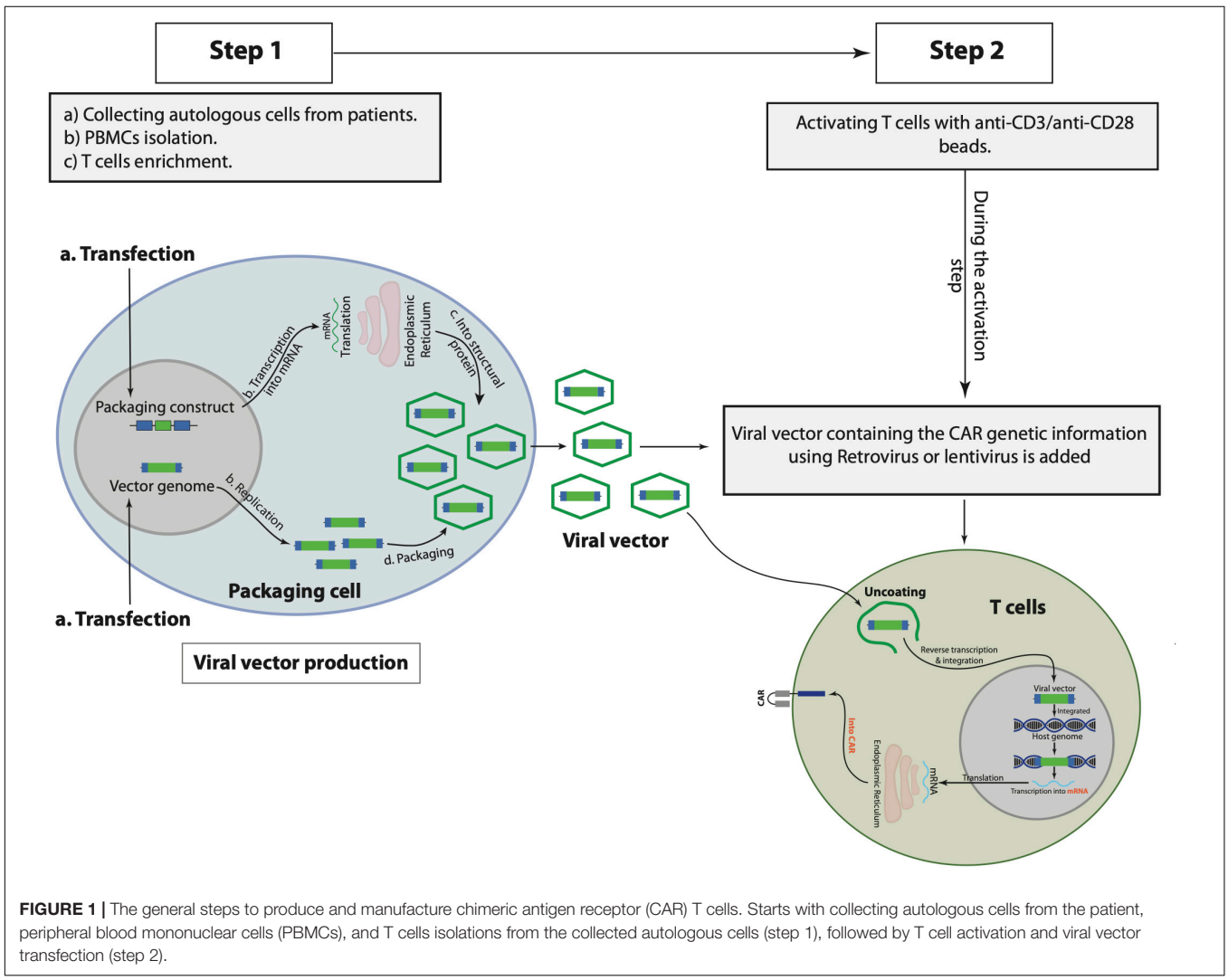

FIGURE 1 | The general steps to produce and manufacture chimeric antigen receptor (CAR) T cells. Starts with collecting autologous cells from the patient, peripheral blood mononuclear cells (PBMCs), and T cells isolations from the collected autologous cells (step 1), followed by T cell activation and viral vector transfection (step 2).

TABLE 2 | The advantages and disadvantages associated with the approaches to produce chimeric antigen receptor (CAR) T cells.

Approach to generate CAR T cells	Main advantages	Main limitations
Viral lentiviral vector	• High transgene expression. • High transduction efficiency. • Persistent gene transfer. • Integrate genetic materials stably into host genome. • Well established system.	• Expensive. • May induce oncogenesis. • Low inflammatory potential. • Has to be tested for safety to ensure the absence of virus replicating competent. • Requires cells pre-activation. • May induce low level of mutagenesis.
Transposon	• Inexpensive. • Safer than viral vectors (lower genotoxicity and less immunogenetic). • Stable genetic integration.	• Low transduction efficiency. • Still under development. • Unknown potential for mutagenesis • Remobilization of the transposons.
mRNA transfection	• Transfect resting nonproliferating cells. • Do not integrate into host genome, therefore associated with very limited mutagenesis and no genotoxicity. • The easiest and the safest.	• Unstable transient expression, therefore requires several cycles of treatment (low transgene expression).

and light regions of a tumor-specific antibody (Zhang C. et al., 2017; Ti et al., 2018). A linker that is flexible and attached via a spacer to the transmembrane domain separates the variable light and heavy chain of the scFv (Zhang C. et al., 2017). The process of CAR development witnesses several evolutions dividing the CAR into five generations, with each generation showing some genetic modifications in their intracellular domain (**Figure 2**). The intracellular domain of the first generation of CAR contains CD3ζ domain only (Tokarew et al., 2019), while the intracellular domain of the second generation is composed of CD3ζ plus costimulatory domain such as CD28 or 4-1BB to improve CAR T cell proliferation and cytotoxic capability (Finney et al., 1998; Hombach et al., 2001; Acuto and Michel, 2003). The third CAR generation has a similar intracellular domain to the second generation with an additional costimulatory molecule to contain two costimulatory molecules instead of one, such as CD28 plus CD137 or CD134 (Zhang C. et al., 2017). The fourth generation is also based on the second generation but replacing the additional costimulatory molecule of the third generation with protein inducer such as IL-12 (Tokarew et al., 2019). The fourth CAR generation was genetically produced to overcome the immunosuppressive microenvironment induced by tumor (Tokarew et al., 2019). IL-12 is capable of inducing IFN-γ as well as granzyme B and perforin by T cells; moreover, it has the ability to inhibit Treg proliferation (Kubin et al., 1994; Cao et al., 2008). Therefore, having IL-12 to be expressed upon CAR T cell activation increased CAR T cells' anticancer activity. A fifth CAR generation based on the second generation is under development to include IL-2 receptor β-chain domain and binding site for STAT3 (Tokarew et al., 2019). Activating CAR T cells through the newly designed scFv provides the three signals that are required for T cell activation such as TCR signal via the CD3ζ domain, costimulatory signal through CD28 domain, and cytokine signaling via the IL-2 and STAT3 domains (Kagoya et al., 2018). CAR T cell activation via their scFv initiates cascade of signaling pathways. The most important three signaling pathways involved with CAR T cell activation includes CD3ζ, CD28, and CD137 signaling that are discussed below.

The intracellular signaling event following CAR binding to the target CSC antigen is the clustering of CAR intracellular domain, as well as the phosphorylation of the three immunoreceptor tyrosine-based activation motif (ITAM) residues of the CD3ζ and the recruitment of the subsequent downstream signaling proteins (Cantrell, 2002; Su and Vale, 2018). The phosphorylated ITAM of the CD3ζ domain interacts with the kinase, CD3ζ-associated protein kinase of 70,000 MW (ZAP70) (Hamerman and Lanier, 2006). In TCR-activated T cells, ZAP70 interaction with phosphorylated ITAM induces major configurational changes in ZAP70 that leads to their consequent interaction with lymphocyte-specific protein tyrosine kinase (Lck), which facilitate ZAP70 phosphorylation and full activation (Williams et al., 1999; Brdicka et al., 2005; Klammt et al., 2015). The fully activated ZAP70 is released from TCR/CD3 complex to the cell plasma membrane to phosphorylate its substrates such as linker for the activation of T cells (LAT) and the SH2-domain-containing leukocyte protein of 76,000 MW (SLP-76) (Katz et al., 2017). The phosphorylated LAT/SLP-76 subsequently partner up with phospholipase C-γ1 (PLCγ1) forming LAT/SLP-76 signalosome and the eventual T cell activation, proliferation, as well as differentiation (Tomlinson et al., 2000). However, the signaling pathway involved in CAR T cell activation via CD3ζ is not fully clear, but it is suggested to rely on the interaction between ZAP70 and CD3ζ ITAM (Mukhopadhyay et al., 2013; Ngoenkam et al., 2018; Ti et al., 2018).

The signals mediated via the costimulatory domain of CAR upon CAR T cells binding to antigens are mainly to improve CAR T cell functionality. CAR binding to specific antigen not only induce ITAM phosphorylation but also the phosphorylation of the tyrosine residues of the CD28, which is included in the intracellular CAR domain (Alegre et al., 2001). The phosphorylation of CD28 domain is mediated by PI3K, followed by growth factor receptor-bound protein 2 (Grb2) recruitment, protein kinase B (PKB/Akt) activation, and the eventual IL-2 production (Alegre et al., 2001; Oberschmidt et al., 2017). The third generation of CAR cells were genetically improved to include additional costimulatory domain such as CD137 to enhance the cell proliferation and survival (Pule et al., 2005; Wang et al., 2007). CD137 is expressed on activated T cells, and upon binding to its legend, the TNFR-associated-factor (TRAF) family including TRAF-1, TRAF2, and TRAF3 are recruited to the CD137 intracellular domain engaging several proteins forming CD137-signalosomes, promoting T cell survival and proliferation (Zapata et al., 2018). Although it has been reported that the functionality of CD137 included in CAR depends on TRAF-1, TRAF2, TRAF3 as well as NF-κB activation

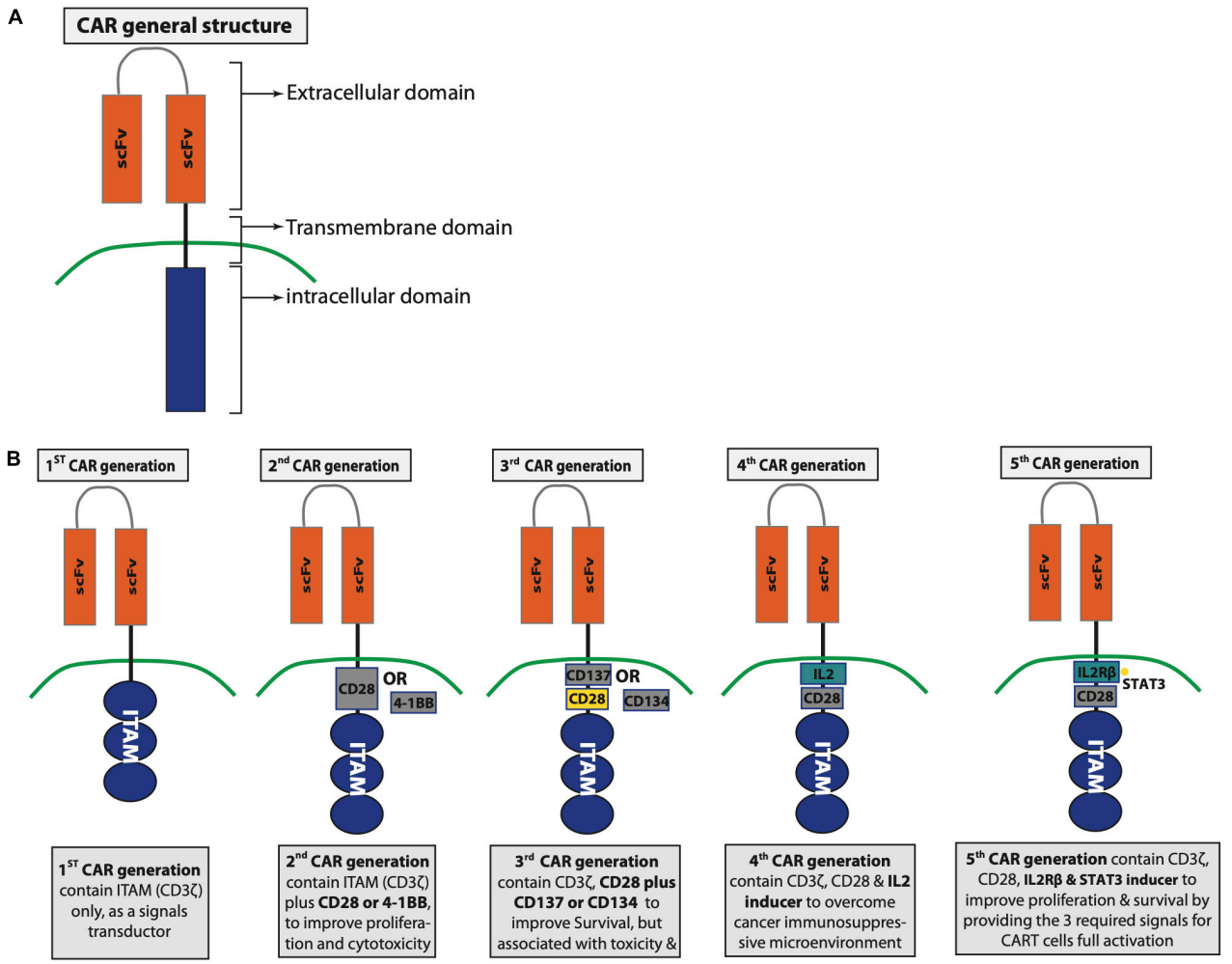

FIGURE 2 | Chimeric antigen receptor (CAR) structure and generations. **(A)** General CAR structure. **(B)** Differences between the developed CAR generations.

(Li G. et al., 2018), however, it is not fully clear if CD137 associated with CAR undergoes a similar response controlling molecular mechanism as CD137 of naive T cells (Zapata et al., 2018). It is extremely important to understand the controlling mechanism of CD137 signaling since it has been reported that signaling derived from CD137 domain of tonic CAR T cells leads to T cell toxicity due to the continues activation of NF-κB by TRAF2 as well as an increase in Fas killing mechanism (Gomes-Silva et al., 2017). However, the CD137 domain plays a key role in improving CAR T cell survival and efficacy, but it has to be considered that unrestricted CD137 activation may be harmful to the cells.

CAR T Cells in Targeting CSCs and Cancer Cells

Although CAR T cells as an immunotherapy in ALL and chronic lymphoid leukemia (CLL) is promising, to date, no CAR T cell targeting CSCs have been approved. As with any treatment, CAR T cells are associated with several advantages as well as disadvantages. The most common advantage with using CAR T cells includes their ability to specifically lyse the target cells independently of MHC molecules, however, CAR T cell treatment could be associated with toxicity, CRS, and soluble tumor syndrome (Guo et al., 2018). To date, very limited number of reports, mostly in animal models, have been published on CSC targeting by CAR T cells. As mentioned above, several antigens have been identified to target CSCs by CAR T cells such as CD133, EpCAM, CD90, and much more (Guo et al., 2018). The pre-clinical and clinical trials as well as the most attractive markers for targeting by CAR T cells are discussed below in terms of relevance and features influencing CAR T cell efficiency.

Preclinical studies testing CSC-specific CAR T cell efficiency, cytolytic activities, and CAR molecule expression must be performed before utilizing these cells as a therapy. For this purpose, xenograft models have been used to evaluate CAR T cells *in vivo*, including line-derived xenograft (CDX), patient-derived xenograft (PDX) models, and models where fresh patient tumor tissues are transplanted into immunodeficient mice (Julien et al., 2012; Rosfjord et al., 2014). A study by Zhu et al. (2015), has

found that CSCs isolated from GBM patients were successfully killed by anti-CD133 CAR T cells both *in vitro* and *in vivo* models of orthotopic tumor. However, CAR T cells upon their direct interaction with glioblastoma stem cells that express CD57 become functionally impaired due to the terminal effect of CD57 on T cell differentiation (Zhu et al., 2015). Moreover, Deng et al. took the lead in generating anti-EpCAM CAR cells to target prostate CSCs. In the latter study, two lines of different tumors were used: PC3 that expresses low levels of EpCAM and PC3M that express high levels of EpCAM. In their settings, PC3M cells were eliminated upon using anti-EpCAM CAR cells *in vivo* and *in vitro*. Although PC3 express low levels of EpCAM, anti-EpCAM CAR cells were able to inhibit the tumor growth of PC3 cells and to prolong the animal survival (Deng et al., 2015). Subsequent study has shown that CAR T cells targeting EpCAM on human ovarian and colorectal cancer cells are capable of killing the cancer cells *in vitro*, and the adaptive transfer of these CAR T cells prolonged the animal survival by eliminating the established ovarian xenografts (Ang et al., 2017). In agreement with these studies, a recent report has documented that the adoptive transfer of CAR T cells targeting cells expressing EpCAM significantly downmodulated the cancer growth in the xenograft model with high level of safety and no associated toxicity (Zhang et al., 2019). A generation of CAR T cells targeting EGFR were engineered by Li H. et al. (2018), which, upon testing, showed antitumor as well as expansion capabilities *in vitro* and prolonged the survival of immunodeficient mice bearing human lung cancer cells, by reducing the cancer tumor burden with no associated toxicity. In the same year, Dong et al., have also generated CAR T cells specific for EGFR but have tested their preclinical capability for hypopharyngeal squamous cell carcinoma *in vitro* only. In their setting, they have found that their generated EGFR-CAR T cells have high cytotoxic potential compared to their control cells with a lysis rate of 52.66% (Dong et al., 2018). Although most of the preclinical trials have shown a success in using CSC-specific CAR T cells by either prolonging the animal's survival, inhibiting the tumor growth, or both, clinically, the success of CAR T cells in solid tumors was limited to feasibility with minimal efficiency due to several factors such as CSCs plasticity and heterogenicity in patients. For example, in clinical oncology, two patients of the same tumor subtype can behave differently to treatment due to their genetic differences leading to interpatient heterogeneity. However, more investigations are required to overcome all the obstacles associated with using immunotherapeutic approaches in solid cancers.

CD133 has been identified as one of the most abundant surface antigens that are highly expressed on several types of cancer CSCs including liver, brain, ovarian, lung, colorectal, and gastric (Yi et al., 2008; Baba et al., 2009; Hibi et al., 2010; Alamgeer et al., 2013; Yamashita and Wang, 2013; Zhang et al., 2014). Moreover, clinical studies have shown that CD133 expressions are extremely associated with disease resistance to treatment and poor prognosis (Zhang et al., 2010; Dragu et al., 2015). Nevertheless, the reports regarding CD133 suitability as CSC marker for certain tumors are still conflicting (Beier et al., 2007; Wang et al., 2008; Barrantes-Freer et al., 2015; Brown et al., 2017). For instance, in GBM, CD133 expression on CSC has been controversial (Bradshaw et al., 2016). It has been reported that human $CD133^+$ GBM cells are capable of initiating brain tumor upon their transfer into immunodeficient mice (Singh et al., 2003, 2004). However, it was also found that $CD133^-$ stem-like cells possessed similar potential of growing tumor successfully in a xenograft model (Beier et al., 2007; Shmelkov et al., 2008; Wang et al., 2008). The fact that CD133 is highly expressed in many cancers, plus it was found to be overexpressed in 50% of HCC, pancreatic, and gastric cancer patients (Ferrandina et al., 2009; Schmohl and Vallera, 2016), and highly expressed with poor prognosis, particularly in HCC (Kohga et al., 2010; Yang et al., 2010) have made $CD133^+$ cells an attractive target for immunotherapy using CAR T cells. Targeting CD133-expressing CSCs with CAR T cells, regardless of the limitations stated earlier, would be of a great potential, however, few studies have investigated anti-CD133 CAR T cells in eliminating CSCs and treating cancer. A study has reported phase I trial using CD133-CAR T cells as antitumor for 23 patients of different cancers, including patients with HCC, pancreatic and colorectal cancers. The trial outcomes were reported between partial remission and stable disease with controlled toxicity (Wang et al., 2018).

Another highly expressed surface marker on many CSCs of several caner types is CD90 (Sukowati et al., 2013; Tang et al., 2013; Zhu et al., 2014; Khan and Mukhtar, 2015; Wang et al., 2015; Woo et al., 2015). CD133 and CD90 share many features including the crucial role in CSC self-renewal, CSC differentiation, and growth (Sukowati et al., 2013; Guo et al., 2018). Moreover, they regulate the oncogenesis of numerous carcinogenic diseases (Sukowati et al., 2013; Guo et al., 2018). In GBM, CD90 has been used for years as a marker for GBM CSCs (Kang and Kang, 2007; Tomuleasa et al., 2010; He et al., 2012; Nitta et al., 2015). However, CD90 expression was not found to be restricted to CSCs of GBM; it is also expressed by mesenchymal stem-cell-like pericytes, GBM-associated stromal cells, tumor-migrating cells, tumor-associated endothelial cell, neuronal cells, and by differentiated GBM cells (Clavreul et al., 2012; Ochs et al., 2013; Avril et al., 2017; Darmanis et al., 2017; Sauzay et al., 2019). Regardless of the high and consistent expression of CD90 in several cancers, CD90 expression on the CSCs of certain tumors has been controversial, particularly in renal cancer. Although CD90 is highly expressed in CSCs expressing CD105 in renal cancer, it is not detected in CSCs of patients with clear renal cell carcinoma (Bussolati et al., 2008; Galleggiante et al., 2014; Khan et al., 2016). However, high expression of CD90 in the CSCs of various cancers, including liver cancer, could be a reason to target $CD90^+$ cancer cells by CAR T cells; unfortunately, no studies using anti-CD90 CAR T cell as a potential treatment for cancer have been reported.

High expression of EpCAM has been reported to play a key role in breast, head and neck squamous cell carcinoma, and colon cancers progression, as reported with CD133 and CD90; EpCAM is crucial for CSC proliferation, differentiation, and renewal (Visvader and Lindeman, 2008; van der Gun et al., 2010). Moreover, EpCAM is reported to be involved in the spread of breast as well as retinoblastoma cancers (Osta et al., 2004; Mitra et al., 2010). In HCC, several studies have shown that

EpCAM is enriched in CSCs of HCC origin and that EpCAM-expressing HCC cells share more stem cell characteristics, have greater invasive, as well as tumor formation ability compared with EpCAM-negative cells (Schmelzer and Reid, 2008; Yang et al., 2008; Yamashita et al., 2008, 2009; Kimura et al., 2010; Li et al., 2016). EpCAM is also overexpressed in colorectal CSC, and it is commonly used with CD44 to identify colorectal CSCs (Dalerba et al., 2007; Liu D. et al., 2014). Several studies have reported that leucine-rich-repeat-containing G protein coupled receptor 5 (Lgr5) can be added to improve the identification panel of colorectal CSCs (Kemper et al., 2012; Jiang et al., 2016). Although EpCAM was also reported to be overexpressed in some types of cancers including breast, prostate, and pancreas, it was not detected in CSC of other cancers such as GBM (Macarthur et al., 2014). A Chinese trial has been conducted using EpCAM-CAR T cells on patients with liver cancer (Liu et al., 2017; Zhang et al., 2019). However, most of the trials are ongoing, and to date, no documented report has been published.

Several studies have reported that cancer cells that have undergone epithelial-to-mesenchymal transition possess more stem-cell-like characteristics, express an increased level of CD44 (Mani et al., 2008), and require CD44v switch to CD44s isoform (Brown et al., 2011; Zhao et al., 2016). Moreover, multiple studies have documented that CD44v expression is associated with metastasis and poor prognosis of several types of solid cancers (Mulder et al., 1994; Kaufmann et al., 1995; Li et al., 2014; Ni et al., 2014; Ozawa et al., 2014; Todaro et al., 2014). In agreement with CD44s and CD44v roles, it was found that increased levels of CD44v, in particular CD44v6, is associated with pancreatic cancer metastasis and more restricted to the late clinical stages of the disease (Rall and Rustgi, 1995; Castella et al., 1996). CD44v6 was stained positive in 50% of tissues isolated from pancreatic cancer patients, while 38% of the tissues obtained from 42 separate patients were positive for CD44v2 but not detectable in healthy tissues. Moreover, the presence of CD44v6-positive tumor cells in patients with primary cancers had given the patient shorter survival rates compared to patients with CD44v6-negative tumor tissues (Gotoda et al., 1998). CD44s was underexpressed in surgically removed specimens from patients with prostate cancers, however, the other isoforms were overexpressed. Independently, increased expression of CD44v2 was associated with improved recurrence-free rate of survival (Moura et al., 2015). To date, no clinical trial has reported CD44-CAR T cells data to treat solid tumors.

Using $CD44^+$, $CD24^-$, and increased ALDH activity has become the "golden standard" method to phenotype the breast CSCs (Park et al., 2010; Gong et al., 2017; Park et al., 2019). In agreement, tissues from breast cancer patients of triple-negative breast cancer (TNBC), the most aggressive form of breast cancer, showed $CD44^+$, $CD24^-$, and high ALDH1 phenotype compared to the nonTNBC tissues (Honeth et al., 2008; Li et al., 2013; Ma et al., 2014). Moreover, it was found that cancer cells that survive chemotherapeutic approaches in TNBC patients were of $CD44^+$, $CD24^-$, and high ALDH1 phenotype and showed more improved mammosphere-forming capacity (Tian et al., 2018). This similarly applies to lung cancer, where ALDH1 plus several other CSC markers including CD44 and CD133 have been identified as markers for lung CSC, but due to the heterogeneity and plasticity of lung cancer, having a specific marker for lung CSC is difficult. However, several studies have shown a strong positive association of ALDH1 with lung cancers, and inhibiting ALDH1 has led to the downregulation of stemness-related genes associated with lung cancer (Jiang et al., 2009; Leung et al., 2010; Gomez-Casal et al., 2013; Duan et al., 2014; Hardavella et al., 2016; Zakaria et al., 2017). ALDH1 has also been recognized as an CSC marker in head and neck cancer, in which an increased ALDH1 activity was associated with enhanced tumorigenesis and greater resistance to chemotherapy. Although ALDH1 has been suggested as a great marker to target CSC by CAR T cells, no study has been reported yet. However, the marker was used successfully to eliminate $ALDH^{bright}$ cells obtained from various cancer cell lines including head and neck, breast, and pancreatic cancer lines *in vitro* with ALDH1A1-specific $CD8^+$ T cells. Upon adoptive transfer of ALDH1A1-specific $CD8^+$ T cells into xenograft-bearing immunodeficient mice, $ALDH^{bright}$ cells were selectively eliminated, cancer growth and metastases were inhibited, and animals' survival were prolonged (Visus et al., 2011). The same approach was also used by Luo et al. (2014) in which $ALDH^{bright}$-specific $CD8^+$ T cells were generated ensuing the inhibition of lung tumor cell line growth as well as prolonging the animal survival.

As discussed earlier, EGFR, in particular $EGFR^{VIII}$, is rarely expressed in healthy tissues, characterizing this exclusive tumor-mutated receptor as an attractive therapeutic molecule. Emlet et al. (2014), have characterized GB CSCs as $EGFR^{VIII+}/CD133^+$ cells with self-renewal as well as cancer initiation capabilities. Moreover, they have found that $EGFR^{VIII+}/CD133^+$ cells can maintain $EGFR^{VIII+}/CD133^+$ phenotype and stem-like characteristics in tumor sphere culture, but not in standard cell culture. $EGFR^{VIII}$ was also found to be coexpressed with undifferentiated cell markers, and upon eliminating $EGFR^{VIII+}/CD133^+$ cells by antibodies of bispecific property in tumor-bearing mice, the tumor generation was inhibited and the mice survival was significantly prolonged (Emlet et al., 2014). For all of the above-mentioned appealing reasons, $EGFR^{VIII}$ was targeted by CAR T cells in patients with $EGFR^{VIII+}$ recurrent GBM; this first clinical trial was done in 10 patients who had been on different therapeutic regimes prior to receiving $EGFR^{VIII}$-CAR T cells. Although one patient on the trial has not shown the need for any further therapies for more than 18 months postreviving CAR T cell infusion, no noticeable tumor regression has been reported by MRI in any of the other patients. This might be due to the high heterogeneity of $EGFR^{VIII}$ expression as well as the presence of tumor immunosuppressive microenvironment, which was worsen by postCAR T cells infusion (O'Rourke et al., 2017). Furthermore, the outcome of an additional study by Goff et al. (2019) on 18 patients with recurrent GBM who had different therapeutic interventions prior to receiving their $EGFR^{VIII}$-specific CAR T cell infusions was not successful, a harbinger of additional barrier in using CAR T cells for treating patients with solid cancers. Moreover, Feng et al. (2017), have tested CAR T cells targeting both EGFR and CD133 to treat one patient with cholangiocarcinoma. Upon the initial infusion of EGFR-CAR T cells, the patient showed partial response of 8.5

months and extra 4.5 months upon receiving CD133-CAR T cells. However, their treatment where associated with CAR T-EGFR resistance and some degree of toxicity, suggesting that regardless of the effectiveness seen, more investigations to improve the adverse side effects are needed (Feng et al., 2017).

Regardless of the initial failure seen upon using CAR T cells to treat metastatic solid tumors, several subsequent studies have confirmed the efficiency of infused CAR T cells in treating primary as well as metastatic tumors. One of the first clinical trials to examine CAR T cells was done to treat metastatic renal cell carcinoma by generating carbonic anhydrase IX (CAIX)-specific CAR T cells. Although the patients enrolled in the study have shown moderate antitumor activity as well as initial tolerance to treatment, upon several infusions, patients showed an increase in their liver enzymes, and due to the toxicity associated, the therapy was ceased (Lamers et al., 2006, 2013). Subsequently, Morgan et al. (2010), used CAR T cells to target HER2 in treating a patient suffering from metastatic colon cancer; however, the treatment was associated with fatal toxicity. Nevertheless, local delivery infusions of IL13Rα2-specific CAR T cells into a patient with recurrent GBM showed no toxic side effects and was associated with the regression of the primary as well as the metastatic spine tumors for 7.5 months. Although none of the initial primary or metastatic tumor recurred, the patient develop tumor at several new locations after a while. This was justified by some preliminary data showing that the new locations possess reduced expression of IL13Rα2 (Brown et al., 2016). The locally infused CAR T cells' potential to prevent adenocarcinoma liver metastases (LM) was also tested by targeting carcinoembryonic antigen (CEA), a protein overexpressed in most epithelial cancers. The study included six patients who received CEA-CAR T cells with/without IL-2 supplement. Among the patients, five died from progressive disease, while one of them survived with a stable disease for 23 months posttreatment, however, all six patients have tolerated the treatment without signs of toxicity. Moreover, biopsies from some of the patients showed an increase in LM necrosis, and patients who received combined therapy documented 37% decrease in their CEA serum levels (Katz et al., 2015). Preclinical studies testing CAR T cell efficiency against metastatic cancers include a recent study showing that local infusion of CAR T cells specific for HER2 into orthotopic xenograft models has high antitumor activities against breast to brain metastases (Priceman et al., 2018). Additional preclinical study in pulmonary xenograft models has shown that vascular endothelial growth factor receptor-1 (VEGFR-1)-CAR T cells coexpressing IL-15 are able to prevent pulmonary metastasis (Wang W. et al., 2013). In a lung cancer model, CAR T-cell-targeting tissue factor (TF), found to be overexpressed in squamous cell carcinoma and adenocarcinoma of nonsmall cell lung cancer as well as melanoma, suppressed the cancer in the xenograft and prevented the metastasis of TF-expressing tumor cells without associated toxicity (Zhang Q. et al., 2017). Recently, Seitz et al. (2020) have generated CAR T cells targeting disialoganglioside GD2, a breast CSC marker, and reported that their generated CAR T cells are capable of preventing the tumor progression as well as the formation of lung metastasis in an orthotopic xenograft model of TNBC. Few studies have been published reporting the efficiency of CAR T cells in preventing metastatic prostate cancer (mPCa) mainly by targeting prostate-specific membrane antigen (PSMA), which is expressed in prostate cancer cells. In a preclinical setting, Zuccolotto et al. (2014), have targeted human PSMA by CAR T cells in prostate tumor-bearing mice, reporting the complete elimination of metastatic cancer cells in majority of the animals. Clinically, Slovin et al. (2013), conducted a phase I trial using CAR T cells specific for PSMA in patients with castrate metastatic prostate cancer. Some patients were stable after receiving the treatment, while others had progressed disease, and the degree of toxicity were dose dependent. Despite all the reported studies and trials, the capability of CAR T cells to prevent metastatic spread still requires more investigations in order to reach applicable clinical conclusions. Moreover, although CAR T cells are a very appealing therapy especially with the incredible success seen in some hematological malignancies, collectively, these data suggest that solid tumor targeting by CAR T cells has a poor efficiency for several reasons and many challenges, which are discussed below. However, there is a great interest in improving CAR T cell efficiency to overcome all the associated issues with their application. **Figure 3** illustrates the possible killing steps by CAR T cells, and **Table 3** summarizes examples of the published clinical trials of CAR T cells in some of the solid tumors.

Barrier in Using CAR T Cells

CAR T cells have revolutionized the world of fighting cancers by immunotherapeutic approaches. Since the reported success of anti-CD19 CAR T cell in treating ALL and CLL and approval of the first anti-CD19 CAR T cells therapy to treat B cell ALL and diffuse LBCL by the Food and Drug Administration (FDA), the number of clinical trials targeting several antigens other than CD19 using CAR T cells has dramatically increased (Kochenderfer et al., 2010a; Kalos et al., 2011; Porter et al., 2011; Mullard, 2017; Tang et al., 2018; Shah and Fry, 2019). However, about 30–50% of patients who received anti-CD19 CAR T cells have relapsed 1 year from their remission, while 10–20% of the patients did not reach the remission phase following anti-CD19 CAR T cell treatment (Lee et al., 2015; Gardner et al., 2017; Maude et al., 2018; Park J.H. et al., 2018). Patients' relapse following treatment with CAR T cells was not exclusive to anti-CD19 CAR T cells, as other approaches using CAR T cells, for example, to target CD22 were also associated with relapse (Fry et al., 2018). This suggest that relapse and recurrence will be a common issue associated with CAR T cell therapy, especially if they were not used to target CSCs.

As mentioned, CAR T cells' potential in treating cancer is very promising, however, the toxicity associated with the treatment is one of the major obstacles. CAR T cell toxicity has been classified into five categories, on-target/on-tumor, on-target/off-tumor, off-target, neurotoxicity, and other toxicities (Sun et al., 2018). On-target/on-tumor is toxicity associated with T cells' release of excessive cytokines as well as the resulted necrotic tumor cell, leading to what is known as CRS and tumor lysis syndrome (TLS), respectively. However, it has been reported that this type of risk can be minimized based on the disease burden and the appropriate monitoring

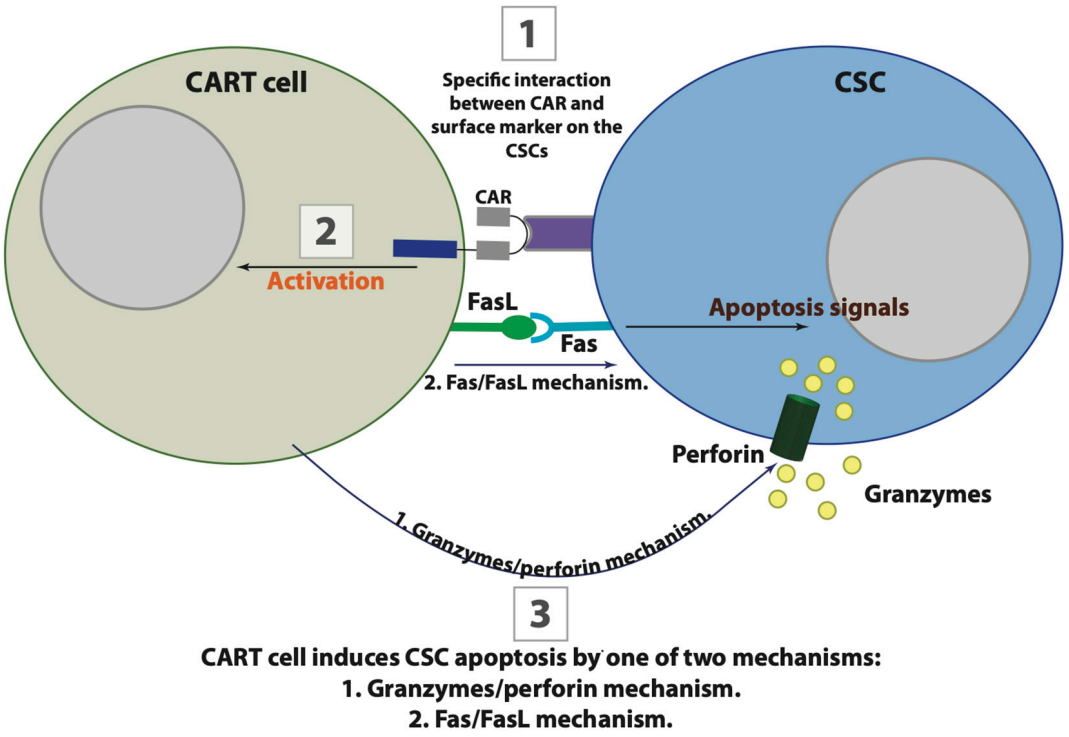

FIGURE 3 | The possible interaction between chimeric antigen receptor (CAR) T cells and cancer stem cells (CSCs). CAR T cells target CSCs in three possible steps that are initiated by CAR binding to their specific antigenic target on CSC (1), followed by CART cells activation (2), and the eventual apoptosis of CSC by one of two killing mechanisms including Fas-FasL or granzymes/perforin (3).

TABLE 3 | Examples of the published clinical trials of chimeric antigen receptor (CAR) T cells in some of the solid tumors.

Tumor type	CSC markers	CAR T cells clinically	Results obtained clinically	References
Brain	CD133 $EGFR^{VIII}$	$EGFR^{VIII}$-CAR T cell	Showed success in one patient, while the others have no noticeable tumor regression.	O'Rourke et al., 2017
		$EGFR^{VIII}$-CAR T cell	Not successful outcomes	Goff et al., 2019
		IL-13Rα2-CAR T cells	Regression of primary and metastatic spine cancer, with no toxicity, but recurrence at several new locations.	Brown et al., 2016
Prostate	EpCAM CD44 ALDH	PSMA-CAR T cells	Mixed outcomes between stability and progressed disease (toxicity was dose dependent)	Slovin et al., 2013
Colon	EpCAM, CD44, Lgr5 CD133 ALDH HER2	HER2-CAR T cell	Fetal toxicity	Morgan et al., 2010
Liver	CD133 EpCAM EGFR CD44 CD90	CD133-CAR T cells (HCC, pancreatic, and colorectal cancers)	Outcomes between partial remission and stable disease with controlled toxicity.	Wang et al., 2018
		EGFR-CAR T cells plus CD133- CAR T cells	EGFR-CAR T cells infusion showed partial response of 8.5 months and extra 4.5 months upon receiving CD133-CAR T cells, with some degree of toxicity.	Feng et al., 2017
		CEA-CAR T cells ± IL-2 supplement	One patient survived and the rest died, however, no toxicity reported.	Katz et al., 2015

as well as the suitable splitting of the doses. Since those risks are rapid immune responses of massive cytokine release, administrating a dose of corticosteroids as well as antagonist mAb can be effective (Brentjens et al., 2013; Teachey et al., 2013; Davila et al., 2014; Maude et al., 2014a; Bonifant et al., 2016; Sun et al., 2018). The most noticeable CAR T-cell-associated toxicity is due to the presence of the target CAR T cell antigen on both the tumor as well as the healthy tissues, a phenomenon known as "on-target/off-tumor" (Sun et al., 2018). This shared expression is enormously damaging because CAR

T cells can target healthy tissues expressing even the lowest levels of the target antigen (Sun et al., 2018). This was seen in an early study performed at Erasmus University, where they have observed that infusing carbonic anhydrase IX-CAR T cells into patients with renal cell carcinoma resulted in cholestasis due to the physiological expression of the target antigen on the epithelial cells of the bile duct (Lamers, 2009; Lamers et al., 2013). These results were not limited to the latter study (Hombach et al., 2010); therefore, selecting target antigen for CAR T cells with the knowledge of its background expression is the most crucial to have better application as well as to decide on the threshold causing toxicity and to determine the possible severity in human (Sun et al., 2018). Recently, a novel universal CAR (uniCAR) system is developed to reduce the risk associated with on-target and to control CAR T cell reactivity, allowing CAR T cell to switch on and off in controlled approach. UniCAR system signaling and antigen-binding characteristics are separated into two independent components. T-cell-expressing uniCAR specifically recognizes human nuclear protein and consists of 10 amino acids; therefore, uniCAR cells are inactive upon infusion due to the lack of their target. UniCAR cells become activated via a separated system that bridge the uniCAR cell binding domain with its nuclear antigen motif fused to tumor-antigen-specific scFV (Cartellieri et al., 2015). Unfortunately, the use of immunodeficient model is insufficient and associated with several drawbacks that limit the assessment of toxicity such as on-target/on-tumor and on-target/off-tumor (Kochenderfer et al., 2010b). One of the challenges associated with immunodeficient model is that human-specific CAR T cells can lead to graft-versus-host disease in mice due to recognizing the mouse xeo-antigens limiting the utilization of this model in evaluating therapies targeting slow-developing cancers without understanding the practical therapeutic window for the model (Alcantar-Orozco et al., 2013). An additional obstacle associated with this kind of animal model is that the mice do not represent the clinical situation due to their limited endogenous lymphocytes. Although cancer patients usually undergo lymphocyte depletion regimens, their lymphocyte recovery occurs, developing the various populations of T cells including Tregs that downregulate the antitumor effect accompanied with the transferred CAR T cells, a situation that is not replicated in the mice model (North, 1982; Gattinoni et al., 2005). However, this model has been useful in confirming that CAR T cells are able to target tumors; the obstacles associated with solid cancer microenvironment might be undervalued (Sharpe, 2018). Therefore, animal equivalent products as well as syngeneic tumor models might be more useful in testing CAR T cells' safety and efficacy (Kochenderfer et al., 2010b; Davila et al., 2013). CAR T cells can go out of their way attacking antigens nonspecifically, off-target toxicity; fortunately, this issue of cross-reactivity has not yet been reported upon using CAR T cells. However, it should be kept in mind while developing CAR T cells targeting certain antigens (Bonifant et al., 2016). Of the most serious toxic effects associated with CAR T cell treatment is neurotoxicity, which has been reported for no certain well-defined causative pathophysiology in patients infused with CD19-specific CAR T cells (Sun et al., 2018). Several other CAR T-cell-associated toxicities have been reported, including immunosuppression, immunogenicity, and genotoxicity. However, for more details on toxicity associated with CAR T cell immunotherapy and the possible strategies to overcome it, readers are referred to reference Sun et al. (2018).

Unlike solid tumors, CAR T cells' systematic administration for hematological malignancies was a success because the target was easily reached by CAR T cells. One of the barriers that CAR T cells have to overcome in solid cancers is reaching their target in the tumor site. However, improving CAR T cells' strength for systemic administration is associated with some safety concerns, as documented upon using HER2-specific CAR T cells for therapy. HER2-specific CAR T cells were generated with high-affinity form of scFv that was able to recognize even normal lung cells expressing low levels of HER2 leading to fatal pulmonary toxicity and CRS (Morgan et al., 2010). One of the possible solutions is the local administration of CAR T cells into the targeted tumor bed. For instance, the administration of IL13Ra2-specific CAR T cells intraventricularly shows intracranial and spinal tumor regression in recurrent GBM patients (Brown et al., 2016). Moreover, mRNA-transduced anti-c-Met CAR T cells were examined through intratumoral administration in a clinical trial on patients with metastatic breast cancer, and the treatment was reported to be feasible and was also associated with extensive tumor necrosis at the site of injection as well as inflammation (Tchou et al., 2017). This study was subsequently confirmed, where intraventricular administration of HER2-specific CAR T cells was reported by Priceman et al. (2018) to have more antitumor response in orthotopic xenograft models of brain metastatic breast cancer when compared to intravenous infusions. Another proposed approach is the use of what is called masked CAR (mCAR) T cells, which only get activated and unmasked upon exposure to protease, which is mostly found in the TME, not in healthy tissues. The concept of mCAR T cells was tested through generating mCAR T cells targeting EGFR that were activated against EGFR-expressing cells upon exposure to tumor protease (Han et al., 2017). CAR T cells' inability to reach their target site is mainly due to their failure to track a chemotactic gradient due to chemokine-receptor mismatch; moreover, CAR T cell entry to the tumor site can get blocked by some physical barriers including cancer-associated fibroblast and abnormal vasculature (Hanahan and Coussens, 2012). Additionally, solid tumor usually causes damage to the blood vessels, known as high endothelial venules, which are considered as important entry points for lymphocytes (Ager, 2017). Since chemokines could play a crucial role in CAR T cells' homing to the tumor site, "armored" mesothelin CAR T cells were generated expressing constitutive IL-7 and CCL19. These generated CAR T cells were found to completely increase tumor regression and to prolong the survival of solid tumor-bearing mice (Adachi et al., 2018). Data in this area are still being collected, with very promising results to improve and to overcome the advised side effects that are usually associated with CAR T cell systemic administration as well as toxicity.

CAR T cells as a monotherapy to treat solid tumors was associated with limited efficiency in most of the clinical trials. Therefore, one of the suggested strategies to increase the efficiency of CAR T cell therapy is to combine it with other therapeutic regimes such as chemotherapy and radiotherapy. Several studies have reported that combining CAR T cells with chemotherapy can reduce the disease-associated side effects, improve the recognition of the tumor antigens, and enhance CAR T cell efficiency and persistence (Proietti et al., 1998; Alizadeh et al., 2014; Muranski et al., 2006). This enhanced efficiency was also seen upon combining CAR T cell therapy with radiotherapy. Weiss et al. (2018) have found that combining CAR T cells with radiotherapy enhance T cell infiltration and transport, produce synergistic activity, enhance the presentation of tumor antigen, and increase CAR T cell durability. Multiple reasons might be behind the enhanced efficiencies and persistence of CAR T cells upon combining it with chemotherapy and radiotherapy, including the ability of those therapies to modify TME and to remove immunoregulatory cells facilitating CAR T cells role. Combining CAR T cell therapy was also suggested to be beneficial with checkpoint inhibitor therapy especially for patients who, postreceiving CAR T cell therapy, might experience antigen escape and subsequent CAR T cell failure and recurrent malignancies. However, this was only reported so far to be effective in mice (John et al., 2013). The reported studies on the direct effect of cancer treatment on T cells' cytotoxic capabilities in targeting CSCs are lacking, unlike NK cells, where Luna et al. (2019), have found that bortezomib, a clinically used proteasome inhibitor to treat multiple myeloma as well as mantle cell lymphoma patients, can enhance the targeting of CSCs by NK cells through upregulating NK cells ligands, MICA and MICB expression, as well as MHC class I on the surface of $ALDH^+$ CSCs. These data support the importance of using combined therapy upon transferring CAR cells, with emphasis on the need to study the exact and direct influence of other therapies that would be combined, on CAR T cell capacity in targeting CSCs. Furthermore, most of CAR T cells' clinical trials to target CSCs have been done on patients who have failed to respond to their therapeutic regimes and are with poor physical conditions, which can be the reason behind the failure of CAR T cells as monotherapy. More importantly, it is impossible for CAR T cells as a monotherapy to eradicate heavy burden solid tumors; therefore, using CAR T cell combined with other therapies would improve the value of CAR T cell therapy, particularly if the patients were selected at early stages of the disease to increase the chance of the removal of both CSCs and nonCSCs at once.

Several other reasons have been cited as obstacles to effective CAR T cell treatment; most commonly is due to alteration or loss of the target antigen (Gardner et al., 2016; Jacoby et al., 2016; Fry et al., 2018), inconsistency of CAR T cells, as well as unsuccessful manufacturing (Mueller et al., 2017; Stroncek et al., 2017; Ceppi et al., 2018). Apart from the success reported with CAR T cells in B cell leukemia and lymphoma, no other diseases have documented this achievement with CAR T cells regardless of their wide use as a targeting therapy. Therefore, understanding the limitations of these cells as a therapy and solving the issues associated with their application is crucial to benefit fully from such powerful approach.

CONCLUSION

The fact that CAR T cells can target any molecule in a cell, independently of MHCs, made CAR T cells targeting CSCs very attractive and a powerful tool, particularly for hematological malignances. Unfortunately, most of the clinical trials using CAR T cell to target CSCs in solid tumors have been disappointing due to several challenging barriers, including toxicity, CRS, soluble tumor syndrome, alteration or loss of the target antigen, as well as unsuccessful manufacturing. Therefore, many groups have tested several strategies to overcome these issues, for example, infusing CAR T cell locally instead of systemically to improve safety and minimize CAR T cell on-target/off-tumor adverse side effects. Moreover, several steps have been taken to upgrade CAR T cells including the generation of uniCAR T cells. However, using CAR T cells to target CSCs will always be associated with obstacles, unless a stable and unique target is identified to differentiate CSCs from the rest of the tumor as well as healthy cells. CAR T cells' future in targeting CSCs is still under investigation, and many studies are needed to both identify the uniquely expressed targets as well as to improve CAR T cell production and administration regimes.

AUTHOR CONTRIBUTIONS

RA collected data from published studies and wrote the manuscript.

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SET Domain Containing 2 Deficiency in Myelodysplastic Syndrome

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Recent studies have shown that myelodysplastic syndrome's (MDS) progression to acute myeloid leukemia (AML) is associated with gene mutations. SET domain containing 2 (*SETD2*) variants were reported as a risk factor of poor prognosis in patients with AML. However, little is known about the potential contribution of the *SETD2* gene in MDS. In this study, we investigated the roles of *SETD2* gene mutations/variants on clinical features and prognosis in patients with MDS. A 43-gene panel was used for next-generation sequencing in 203 patients with primary MDS, and then the effects of *SETD2* mutation on Wnt/β-catenin signaling was investigated during the different stages of MDS. At a median follow up of 33 months, 65 (32.0%) deaths and 94 (46.3%) leukemic transformations were recorded. The most frequent mutations/variants included *TET2*, *DNMT3A*, and *ASXL1* mutations/variants. 37 patients had *SETD2* gene mutations/variants, and these patients exhibited a significantly increased frequency of *TP53* mutations. Multivariate survival analyses indicated that *SETD2* mutations/variants were closely associated with overall survival (OS), and they were identified as risk factors for progression-free survival (PFS), especially with low expression of *SETD2* gene. Further, we found that *SETD2* loss could promote MDS progression via upregulation *DVL3* mRNA level in BM cells and it could also cause genomic instability. Secondary mutations, such as *TP53* and *FLT3* mutations, were acquired at the time of progression to AML. In conclusion, we showed that *SETD2* deficiency was associated with poor outcomes in patients with MDS. Moreover, *SETD2* deficiency may upregulate *DVL3* expression and modulate genomic stability that caused AML transformation.

Keywords: gene mutation, myelodysplastic syndrome, progression-free survival, SET domain containing 2, *DVL3*

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INTRODUCTION

Myelodysplastic syndrome (MDS) is a clonal myeloproliferative disorder of hematopoietic stem cells (HSCs) and can evolve into aggressive forms of acute myeloid leukemia (AML) (Greenberg et al., 2017). Transformation to AML often involves genetic mutations that can be consistently recognized in MDS. Up to 80% of patients with MDS have one or more gene mutations, and as the number of oncogenic mutations increases, overall survival (OS) and progression-free survival (PFS) progressively worsen (Kim et al., 2017; Tefferi et al., 2017). Some of these molecular markers can be used to predict clinical outcomes in patients with MDS (Visconte et al., 2019; Hospital and Vey, 2020). Epigenetic modifications, particularly aberrant methylation of cancer-related genes

such as Tet methylcytosine dioxygenase 2 (*TET2*) and DNA methyltransferase 3A (*DNMT3A*), are common abnormalities in MDS (Hosono, 2019). The role of epigenetic deregulation has been well-documented and has led to the successful development of epigenetic therapies. Recently, several studies have demonstrated that the methylation of *TET2*, *DNMT3A*, and *DNMT3B* is related to mutations in SET domain containing 2 (*SETD2*), which can drive tumorigenesis by coordinated disruption of the epigenome and transcriptome (Imielinski et al., 2012; Baubec et al., 2015; Tiedemann et al., 2016; Tlemsani et al., 2016).

The tumor suppressor gene *SETD2* is a histone methyltransferase that functions to trimethylated lysine 36 in histone H3. As a transcriptional regulator, *SETD2* has been shown to participate in diverse biological processes including alternative splicing, transcriptional elongation, DNA repair, and embryonic differentiation (Venkatesh et al., 2012; Li et al., 2013; Neri et al., 2017). *SETD2* mutations are often present and predict poor survival in several types of leukemia as well as various solid tumors (Kandoth et al., 2013; Mar et al., 2014; Zhu et al., 2014; González-Rodríguez et al., 2020). A recent study confirms that loss-of-function *SETD2* mutations facilitate the initiation of leukemia and impair DNA damage recognition, leading to resistance to therapy (Sheng et al., 2019). Another study demonstrate that *SETD2* is required for the self-renewal of HSCs and that *SETD2*-deficient HSCs contribute to the development of MDS (Zhang et al., 2018). Nevertheless, the roles of *SETD2* in MDS remain largely unknown. In this study, we investigated the effects of *SETD2* gene mutations/variants on clinical features and prognosis in patients with MDS, which provided insights into the roles of *SETD2* in MDS.

PATIENTS AND METHODS

Patients

All study participants had been diagnosed with MDS according to World Health Organization criteria (Arber et al., 2016). Patients with MDS identified at Ruijin Hospital North, Shanghai Jiao Tong University from May 2015 to December 2019. This report included follow-up data through March 1, 2020, with a median follow-up period of 33 months (range: 3–60 months). OS and PFS were evaluated as disease outcomes, and events were defined as any AML transformation or death. The OS time was calculated from the time of diagnosis to the time of death or to the last follow-up. PFS was defined as the period beginning when the patient was diagnosed with MDS until the time of AML transformation progression, relapse, or death. Informed consent was obtained from all patients, and the study protocol was approved by the Ethic Committees of Ruijin Hospital North, Shanghai Jiao Tong University School of Medicine.

Sample Collection and DNA Extraction

Bone marrow (BM) samples were harvested from all patients and patient-matched germline reference samples such as oral mucosal cells, hair with hair follicles, or peripheral blood lymphocytes (PBMC) were also harvested. BM mononuclear cells were obtained by centrifugation on a Ficoll-Hypaque at a density gradient of 1500 × g for 25 min, and then washed three times in phosphate-buered saline (PBS). Next, 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was added, and samples were stored at −20°C. Normal DNA was obtained from normal tissues or blood samples. Blood DNA was extracted by Qiagen blood extraction kit (Qiagen, Hilden, Germany), and tissue DNA was extracted using FastPure FFPE DNA Isolation Kit (Vazyme, Nanjing, China) following the manufacturer's protocol. DNA Sanger sequencing from patient-matched tissues and PBMC was applied to determine the presence of germline mutations. DNA quality was assessed by agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific,Wilmington, DE, United States).

Targeted Next-Generation Sequencing

Targeted Sequencing was performed with the illumina Hiseq Xten platform at the sequencing laboratory of Tissuebank Precision Medical Co, Ltd. (Shanghai, China). A total of 10 ng DNA per sample was amplified by PCR, and then the library was captured by using xGen® Lockdown® probes and xGen Hybridization and Wash Kit; Illumina Hiseq sequencer carried out pair end sequencing with a depth of 200X. 43 pathogenic genes (*ASXL1*, *BCOR*, *BCORL1*, *BRAF*, *CALR*, *CBL*, *CDKN2A*, *CEBPA*, *CREBBP*, *CSF3R*, *CUX1*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *GATA1*, *GATA2*, *GNAS*, *IDH1*, *IDH2*, *IKZF1*, *JAK2*, *JAK3*, *KIT*, *KRAS*, *MPL*, *NF1*, *NPM1*, *NRAS*, *PHF6*, *PIGA*, *PTEN*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1*, *ZRSR2*) were screened in all patients, including the entire coding regions and exon-intron boundaries. This multi-gene panel was expected to cover 100% of the targeted area. DNA was sheared into short genetic fragments (150∼200 bp) using the Covaris LE220, which included purified and captured gene fragments. Adaptor-ligated amplicons were prepared using the Illumina Paired-End Sample Preparation kit. Illumina multi-PE-adaptors were bound to terminal genes and target enrichment was performed by probe capture, amplicons were purified using VAHTS DNA Clean Beads and captured on the Illumina Hiseq Xten instrument.

Mutation Analysis

Both VarScan and GATK software were adopted for data analysis, including quality assessment, reading comparison, variant identification, variant annotation, visualization, and prioritization. Variant Call Format (VCF) files were annotated with ANNOVAR software, and variants were prioritized using their minor allele frequency of the variant (MAF < 0.01), zygosity, function, location within the gene, and pathogenicity according to ClinVar. MAF was evaluated by data from 1,000 Genomes Project, the Exome Sequencing Project, and the Exome Aggregation Consortium Database. The nature of novel gene mutations was established based on the American College of Medical Genetics and Genomics (ACMG) guideline.

The conservation and deleteriousness of the variants were investigated using ANNOVAR which interrogated the following

tools: SIFT, PolyPhen 2 HVAR, Polyphen2 HDIV, MutationTaster, MutationAssessor, Likelihood ratio test (LRT), FATHMM, MetaSVM, MetaLR, GERP++, PhyloP, VEST3, DANN, CADD, PROVEAN, fathmm-MKL, Integrated_fitCons, SiPhy_29way, and PhastCons. Non-synonymous germline mutations with a frequency > 1% or synonymous gene mutations were filtered out. On basis of a combination of these tools (two of the 19 tools predicting damaging effects) to evaluate potential pathogenic gene mutations/variants, we searched the published literature for selected gene mutation/variant studies to further assess their potential pathogenicity. Variants that meet these criteria and do not exist in the control group were considered destructive. Briefly, altered DNA sequences were deemed as mutations/variants if they were associated with a hematologic malignancy, if they were assessed with potential pathogenicity, or if they were suspected to be related to clinical efficacy and safety.

During the analysis, we used genome-wide association studies (GWAS) and cancer genome Atlas (TCGA) to discover germline and somatic mutant genes related to MDS. Somatic gene mutations were identified by comparing paired tissue and BM. We utilized variant calls from non-tumor control samples to filter germline gene mutations, and blood samples to track the VAF of gene mutations. If germline gene mutations were recognized in an individual with MDS, sanger sequencing would be used to screen other available family members to find the identified gene mutations.

Western Blotting

Total cellular protein was extracted with RIPA lysis buffer (Beyotime; cat. no. P0013B). Protein concentrations were determined using BCA assays. Next, 30 μg protein lysate was separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). Membranes were immunoblotted with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies in PBS containing 0.5% Tween-20 and 5% bovine serum albumin. The following antibodies were used in this study: anti-SETD2 (Santa Cruz Biotechnology, Santa Cruz, CA, United States; cat. no. sc-99451) and anti-β-catenin (Abways; cat. no. CY3523). Western blot signals were obtained by detecting chemiluminescence on a Typhoon FLA 9500 (GE Healthcare, WI, United States). Image J was used to analyze the signal intensities. Each blot shown in the figures was representative of at least three experiments.

Immunofluorescence Analysis

Immunofluorescence analysis was performed using standard procedures. Briefly, cells seeded in 24-well plates were fixed with 4% paraformaldehyde and then permeabilized with 1% Triton. Cells were then incubated overnight at 4°C with anti-SETD2 antibodies (Sigma Aldrich, St. Louis, MO, United States; cat. no. HPA042451) or anti-β-catenin antibodies (Abways; cat. no. CY3523) and then detected the next day with AlexaFluor 647 goat anti-mouse IgG antibodies or AlexaFluor 488 alpaca anti-rabbit IgG antibodies. 4′, 6-Diamidino-2-phenylindole was used to stain the nuclei. Immunofluorescence images were observed on a fluorescence microscope (Leica; cat. no. DMI4000B).

RNA Extraction and Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using RNAiso Plus reagent (Takara, Shiga, Japan), and 1.5 μg total RNA from cultured cells was reverse transcribed using a PrimeScriptP RT Reagent Kit (Takara) according to the manufacturer's instructions. RT-qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The amplified transcript level of each specific gene was normalized to that of actin.

Statistical Analysis

This was a retrospective study, and descriptive statistics were collected at initial diagnosis. Comparison of age and blast cells was analyzed with Student's *t*-test. Hemoglobin difference was analyzed with Mann-Whitney test. The result of *SETD2* mRNA expression was analyzed by Student's t test and a chi-squared test after testing for normality with the Kolmogorov-Smirnov test. Categorical variables were compared using Fisher's exact test or chi-squared test as appropriate. Patient groups with nominal variables were compared by chi-squared test. Survival analysis was considered from the date of diagnosis to date of death or last contact. Survival curves were prepared by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards regression model and multivariate cox proportional hazards models were used to calculate hazard ratios (HRs) with 95% confidence intervals (CIs) of association pertaining to the relationship between risk factors and survival. Statistical analyses were conducted with SPSS software, version 21.0. Statistical significance was determined by log-rank test, chi-squared test, or Fisher's exact test. For all statistical tests, a *P*-value of less than 0.05 was considered significant.

RESULTS

Patient Cohort: Clinical Characteristics

In total, 203 patients with primary MDS, including 137 men and 66 women, were enrolled in this study. The median age at diagnosis was 60 years (range: 30–80 years). The IPSS-R risk distribution was 15.8% very high, 26.1% high, 36.5% intermediate, and 21.7% low. The median bone marrow blasts and hemoglobin were 7% (range: 0.5–19.0%) and 65 g/L (range: 36–109 g/L). OS and PFS were evaluated as disease outcomes, and events were defined as any AML transformation or death. All survival end points were censored at the date of last follow-up when progression or death was not observed. During follow-up, 65 (32.0%) deaths and 94 (46.3%) leukemic transformations were recorded. Patients received treatment with hypomethylating agents (n = 182), induction chemotherapy (n = 87), allogeneic stem cell transplantation (n = 14), and lenalidomide/thalidomide/danazol (n = 10).

Gene Mutations in MDS

At least one mutation/variant was detected in 166 (81.8%) patients; 36.5% harbored three or more mutations/variants. The most frequent mutations/variants included *TET2* (26.1%), *DNMT3A* (19.2%), *ASXL1* (18.2%), *SETD2* (18.2%), SRSF2 (14.8%), *TP53* (13.3%), *SF3B1* (10.8%), *U2AF1* (14.3%). The common gene mutations/variants were detected in *RUNX1* (5.4%), *IDH2* (4.4%), *SETBP1* (3.4%), *JAK2* (4.9%), *CBL* (3.9%), *CEBPA* (3.0%), *ETV6* (2.5%), *IDH1* (1.5%) and *CSF3R* (1.0%). *SETD2* mutations/variants were found in 37 patients (18.2%), including eight single nucleotide variants: p.(M761I), n = 1; p.(E639K), n = 2; p.(P193L), n = 2; p.(M1080I), n = 7; p.(N1155K), n = 15; p.(P1962L), n = 28; p.(L2486R), n = 1; p.(E1142G), n = 1; and two frameshift mutations [p.(T2388fs), n = 1; p.(F1116fs), n = 1]. *SETD2* p.(P1962L) (13.8%), p.(N1155K) (7.4%) and p.(M1080I) (3.4%) were more common in patients with MDS. We evaluated the relationships between *SETD2* and other gene mutations/variants, and found that 37 patients with *SETD2* alterations had at least one other alterations. Notably, they showed significantly more frequent *TP53* gene mutations compared with patients with wild-type *SETD2* (37.8% vs. 7.8%, respectively; $P < 0.001$). Moreover, *SETD2* mutations/variants were associated with higher BM blast content (10% vs. 6%, respectively; $P < 0.001$) and death rates (59.5% vs. 25.9%, respectively; $P < 0.001$). The clinical characteristics of patients with *SETD2* mutations/variants were summarized in **Table 1**.

SETD2 Mutations/Variants Predicted Poor Prognosis in MDS

ASXL1 mutations/variants were of no significance to inferior OS (**Table 2**); *TP53* mutations/variants were related to inferior OS both on univariate analyses [hazard ratio (HR) = 3.5, 95% confidence interval (CI): 1.9–6.2, $P < 0.0001$] and multivariate analyses (HR = 2.7, 95% CI: 1.4–5.0, $P = 0.003$); *SETD2* mutations/variants were also identified as risk factors for inferior OS by both univariate analysis (HR = 2.7, 95% CI: 1.6–4.4, $P = 0.0002$) and multivariable analysis (HR = 2.0, 95% CI: 1.2–3.5, $P = 0.01$). The addition of age risk stratification to the multivariate model did not affect the significance of *SETD2* and *TP53* for inferior OS.

ASXL1 mutations/variants were of no significance to inferior PFS (**Table 2**); *TP53* mutations/variants were related to inferior PFS both on univariate analyses (HR = 2.9, 95% CI: 1.8–4.8, $P < 0.0001$) and multivariate analyses (HR = 2.7, 95% CI: 1.6–4.6, $P = 0.0003$), and the addition of IPSS-R risk stratification to the multivariate model did not affect the significance of *TP53* for inferior PFS. However, *SETD2* mutations/variants were of only borderline significance on univariate analysis ($P = 0.05$). We investigated the conservation and deleteriousness of mutations/variants by using the soft tools (**Supplementary Table 1**) and searching the published study (Wang et al., 2019), We found *SETD2* p.(P1962L) and p.(N1155K) were not considered as damaging. The variant allele frequency (VAF) of *SETD2* were tracked before and after treatment. Following the decitabine administration, it was shown that the VAF of *SETD2* p.(P1962L) (Sample 55, 21% vs. 38%; Sample 46, 100% vs. 39%; Sample 69, 53% vs. 39%; Sample 37, 60% vs. 45%) and p.(N1155K) (Sample 42, 18% vs. 0%; Sample 44, 100% vs. 0%; Sample 84, 51% vs. 32%; Sample 46, 100% vs. 48%) experienced a marked change, which suggested the likely association with therapy outcome in MDS. Therefore, patients with mutations/variants were divided into two groups [Group A, n = 23, only with *SETD2* p.(N1155K) or p.(P1962L); Group B, n = 14, the remaining mutations/variants]. The differences were statistically significant between the two groups on PFS (**Supplementary Table 2**). For Group B, univariate analysis of PFS identified *SETD2* mutations/variants as a significant risk factor (HR = 4.3, 95% CI: 2.3–7.9, $P < 0.0001$), and this factor retained significance during multivariate analysis (HR = 3.0, 95% CI: 1.5–5.8, $P = 0.001$). The addition of IPSS-R to the multivariate model did not affect the impact of *SETD2* on PFS (**Table 3**). In order to better understand the risk-specific prognostic value, we performed additional analyses by grouping

TABLE 1 | Characteristics of patients according to SETD2 mutation status.

Characteristics	*SETD2* mutations/ variants n = 37	*SETD2* wide type n = 166	*P*-value
Age in years, median (range)	62 (33–80)	60 (30–79)	0.177
Hemoglobin, g/L, median (range)	65 (36–99)	67 (36–109)	0.293
BM blast %, median (range)	10 (1–19)	6 (0.5–19)	<0.001
IPSS-R, n (%)			0.173
Very high	4 (10.8%)	28 (16.9%)	
High	12 (32.4%)	41 (24.7%)	
Intermediate	17 (46.0%)	57 (34.3%)	
Low	4 (10.8%)	40 (24.1%)	
TP53 Mutation, n (%)	14 (37.8%)	13 (7.8%)	<0.001
ASXL1 Mutation, n (%)	6 (16.2%)	29 (17.5%)	0.855
Death	22 (59.5%)	43 (25.9%)	<0.001
AML transformation	21 (56.8%)	73 (44.0%)	0.159
OS, months, median (range)	16 (2–60)	22 (1–68)	0.186
PFS, months, median (range)	11 (1–53)	15 (1–64)	0.077

TABLE 2 | Univariate and multivariate analyses of Overall and Progression-free survival in 203 patients with MDS.

Mutations	Univariate *P*-value; HR (95%CI)	Multivariate *P*-value; HR (95%CI)	Multivariate age adjusted *P*-value; HR (95%CI)
	Overall survival		
SETD2	0.0002; 2.7 (1.6–4.4)	0.01; 2.0 (1.2–3.5)	0.03; 1.9 (1.1–3.3)
TP53	$P < 0.0001$; 3.5 (1.9–6.2)	0.003; 2.7 (1.4–5.0)	0.02; 2.2 (1.1–4.2)
ASXL1	0.9; 1.0 (0.5–1.8)	0.8; 1.1 (0.6–2.1)	0.9; 1.0 (0.6–2.0)
	Progression-free survival		
SETD2	*0.05*; 1.6 (1.0–2.6)	0.4; 1.2 (0.7–2.1)	0.2; 1.4 (0.8–2.3)
TP53	$P < 0.0001$; 2.9 (1.8–4.8)	0.0003; 2.7 (1.6–4.6)	0.02; 1.9 (1.1–3.2)
ASXL1	0.6; 0.9 (0.5–1.5)	0.9; 1.0 (0.6–1.6)	0.9; 1.0 (0.6–1.7)

TABLE 3 | Univariate and multivariate analyses of Overall and Progression-free survival in 180 patients with MDS.

Mutations	Univariate P value; HR (95%CI)	Multivariate *P*-value; HR (95%CI)	Multivariate age adjusted *P*-value; HR (95%CI)
Overall survival			
SETD2	0.0005; 3.9 (1.8-8.5)	0.01; 2.9 (1.2–7.0)	0.03; 2.7 (1.1–6.4)
TP53	0.009; 2.7 (1.3-5.6)	0.1; 1.9 (0.8–4.5)	0.2; 1.7 (0.7–3.9)
ASXL1	0.6; 1.2 (0.6-2.3)	0.5; 1.2 (0.6–2.4)	0.9; 1.1 (0.5–2.1)
Progression-free survival			
SETD2	*P* < 0.0001; 4.3 (2.3-7.9)	0.001; 3.0 (1.5–5.8)	*P* < 0.0001; 4.3 (2.2–8.5)
TP53	*P* < 0.0001; 4.0 (2.4-6.8)	*P* < 0.0001; 3.2 (1.8–5.7)	0.01; 2.1 (1.2–3.6)
ASXL1	0.9; 1.0 (0.6-1.6)	1.0; 1.0 (0.6–1.7)	0.9; 0.9 (0.6–1.7)

SETD2 or *TP53* together as adverse mutations/variants for PFS. Kaplan-Meier analysis for PFS identified *SETD2* or *TP53* as significant risk factors ($P < 0.001$, **Figure 1**). However, for Group A, *SETD2* variants were not related to inferior PFS (**Supplementary Table 3**). The most recent study demonstrated that low expression of *SETD2* promoted the transformation of MDS into AML (Chen et al., 2020), which was consistent with our outcomes of RT-PCR. The pretreatment baseline expression of *SETD2* mRNA (Group A, $n = 16$; Group B, $n = 14$) was lower in the two groups than those in *SETD2* mutations/variants absent controls (Group C, $n = 20$), and there was evident difference between the two groups (Group A vs. Group B, $P < 0.001$) (**Supplementary Table 4**). In all, *SETD2* mutations/variants were considered as significant risk factor of poor outcomes in MDS patients, especially with low expression of *SETD2* gene.

SETD2 May Modulate Wnt Signaling by Regulating *DVL3* Expression

Next, we characterized the molecular and genetic abnormalities of a novel variant form in a patient with MDS. DNA sequencing analysis showed a homozygous single-base insertion between nucleotides 3350 and 3351 in the *SETD2* coding sequence (**Figure 2A**). In order to predict and to better understand the functions of the mutated SETD2 protein, the ITASSER server was used to construct the three-dimensional (3-D) structural of this protein (Shanghai Jiao Tong University School of Medicine, Shanghai, China). Protein structural and functional prediction analysis showed this mutation resulted in the deletion of amino acids after amino acid 1116 in SETD2 and the formation of a new truncated SETD2 molecule (**Figure 2B**); however, the truncated SETD2 lacked functional binding sites, which could not work properly (**Figure 2C**).

Accordingly, we then performed western blotting using BM cells from the patient and showed that SETD2 was almost

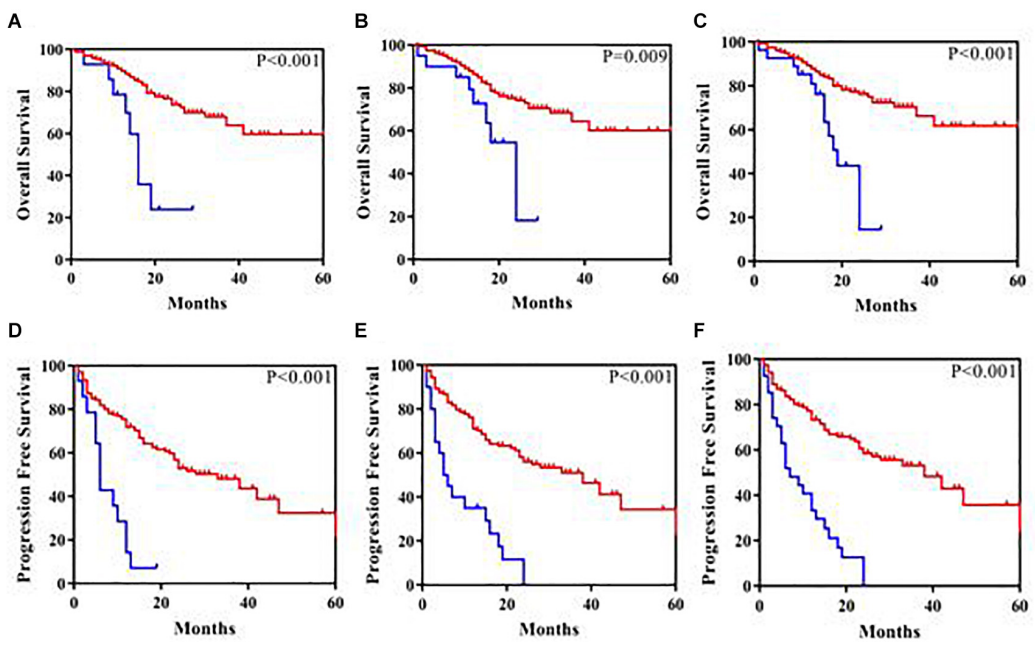

FIGURE 1 | Kaplan-Meier analysis of overall survival and progression-free survival in 180 patients. **(A)** Overall survival according to *SETD2* mutations/variants present (the blue color) compared with *SETD2* mutations/variants absent (the red color). **(B)** Overall survival according to *TP53* mutations/variants present (the blue color) compared with *TP53* mutations/variants absent (the red color). **(C)** Overall survival according to *SETD2* or *TP53* mutations/variants present (the blue color) compared with *SETD2* or *TP53* mutations/variants absent (the red color). **(D)** Progression-free survival according to *SETD2* mutations/variants present (the blue color) compared with *SETD2* mutations/variants absent (the red color). **(E)** Progression-free survival according to *TP53* mutations/variants present (the blue color) compared with *TP53* mutations/variants absent (the red color). **(F)** Progression-free survival according to *SETD2* or *TP53* mutations/variants present (the blue color) compared with *SETD2* or *TP53* mutations/variants absent (the red color).

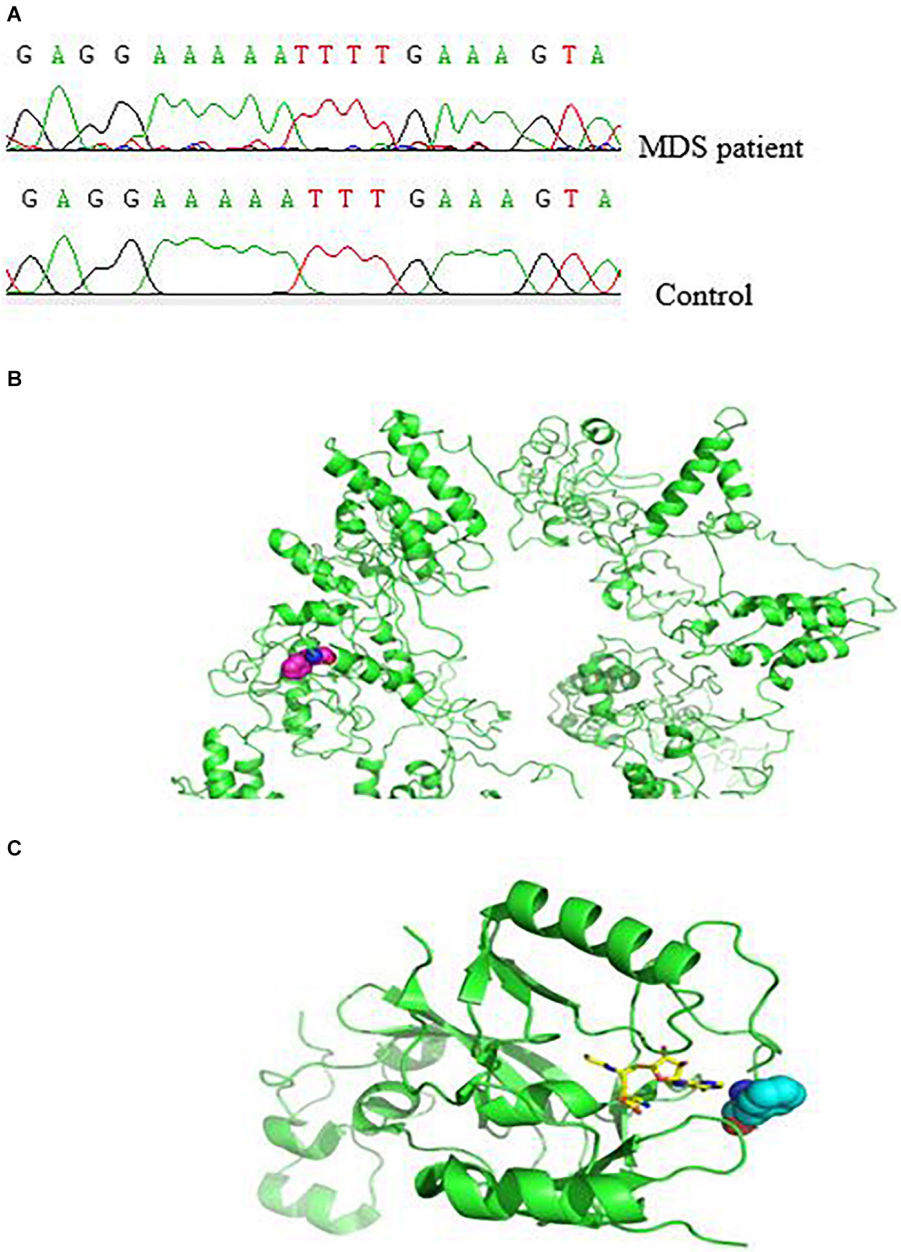

FIGURE 2 | Effect of *SETD2* p. F1116fs in a patient with MDS. **(A)** Part of the *SETD2* gene sequence containing the 3350–3351insT mutation. **(B)** Modeling of the structure of SETD2 protein and location of amino acid mutations. The green color indicates the structure of SETD2 protein, and the red line shows the amino acids changed to terminating codons. **(C)** Locations of amino acid mutations in *SETD2* and functional binding regions. The green color indicates SETD2 protein, the red line shows the amino acids changed to terminating codons, blue lines show functional binding sites, and colored lines show the small molecules. The protein was modeled using I-TASSER and Pymol software.

undetectable when the *SETD2* mutation was identified at initial diagnosis of MDS (**Figure 3A**). Notably, the distribution of β-catenin in BM cell nuclei was significantly increased, as demonstrated by fluorescence microscopy (**Figure 3B**). Similarly, subcellular fractionation and western blot analyses of BM cells from case 6 confirmed that *SETD2* expression was deficient in the nucleus, whereas the level of β-catenin in the nucleus was enhanced compared with that in the cytoplasmic control (**Figure 3C**). RT-qPCR analyses indicated that these BM cells with *SETD2* mutation produced normal levels of *GSK3B*, *APCS*, *DVL1*, and *DVL2* mRNAs, whereas *DVL3* mRNA was upregulated in the absence of *SETD2* (**Figure 3D**). The patient received decitabine combined with AA regimen (aclarubicin, Ara-C); however, it had no response and transformed to AML after two cycles of therapy. He developed severe anemia, and pathological hematopoiesis was found everywhere in bone marrow smears, suggesting

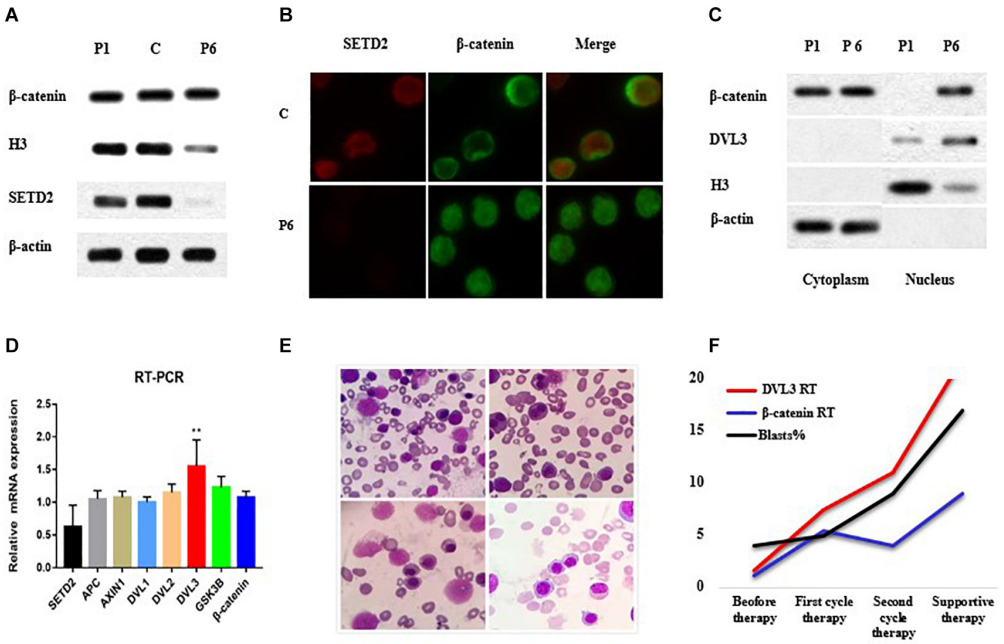

FIGURE 3 | *SETD2* modulated *DVL3* expression to regulate Wnt signaling. **(A–C)** Effects of *SETD2* mutation on the nuclear localization of β-catenin in patients with MDS. **(D)** RT-PCR analysis of Wnt target gene expression in patients with MDS. **(E)** The definitive erythroid development in BM smears. **(F)** The level of gene expression and blasts numbers before and after treatment. C, control; P1, MDS case without *SETD2* mutation; P6, MDS case with *SETD2* p. F1116fs mutation. $^{**}P < 0.05$, the level of *DVL3* mRNA expression was higher than that of control.

the deficient erythroblast differentiation (**Figure 3E**). With the increased numbers of blast cells in BM, the level of *DVL3* and β-catenin mRNA expression improved synergistically (**Figure 3F**), while the protein level of *DVL3* was not increased, suggesting that β-catenin might be indirectly regulated at protein level.

Notably, loss of *SETD2* could cause DNA replication defects and genomic instability. Somatic mutations were successively acquired at the time of progression to AML. These mutations in each chromosome were listed in **Figure 4**. Number and type of newly identified mutations showed a majority of nonsynonymous variants. It should be noted that the new mutations in genes involved in signaling pathway (*FLT3, TP53, TET2, ASXL1*) were identified when the patient with MDS transformed to AML (**Table 4**). Additionally, these additional mutations were accompanied by expansion of existing mutations (*TGF*β p.(P10L), *IL3* p.(P27S), *IL10* p.(R351G). Finally, the patient died of cerebral hemorrhage.

DISCUSSION

In this study, we found that *TET2*, *DNMT3A*, *ASXL1*, and *TP53* were commonly mutated in 203 patients with MDS, consistent with a previous study (Yu et al., 2020). *SETD2* mutations have been detected in a subset leukemia. For example, Non-*MLL* rearranged AML and chronic lymphocytic leukemia exhibit similar incidence rates of *SETD2* mutations (6 and 7%, respectively), and a lower incidence (3%) has been reported in chronic lymphocytic leukemia (Masetti et al., 2016; Parker et al., 2016). *SETD2* mutations/variants were detected in 37 of 203 cases, and new mutations were only found in four cases (2.0%). These studies did identify the rare nature of *SETD2* mutations in leukemia and MDS. *SETD2* mutations/variants often occurred simultaneously with *TP53* mutations in our study. Recently, the *SETD2* gene has been shown to directly regulate the transcription of a subset of genes via cooperation with the transcription factor *TP53* and contribute to further inactivation of *TP53*-mediated checkpoint control (Carvalho et al., 2014; Xie et al., 2008). In addition, *SETD2* mutations have been linked to poor clinical prognosis in various tumors, such as in renal clear cell carcinoma and AML (Wang et al., 2019; González-Rodríguez et al., 2020). Notably, *SETD2* deficiency has been found to impair HSC self-renewal and induce MDS transformation in a conditional *SETD2*-knockout mouse model (Zhang et al., 2018). In our study, we assessed the effects of *SETD2* mutations/variants in patients with MDS, and observed that *SETD2* deficiency was an IPSS-R-independent factor predicting shorter PFS in both univariate and multivariate analyses.

Evidence from human genomes sequencing has linked *SETD2* to MDS, but its causal role has not been reported yet. Previous observation was that *SETD2* gene modulated Wnt signaling by regulating β-catenin (Yuan et al., 2017), and *SETD2* could enhance susceptibility to tumorigenesis in the context of dysregulated Wnt signaling through epigenetic regulation of RNA processing, including *DVLs* (Barry et al., 2013; Wang et al., 2015). Interestingly, our results indicated that *SETD2* loss could promote MDS progression via upregulation of *DVL3* in a patient harboring *SETD2* p.(F1116fs) mutation.

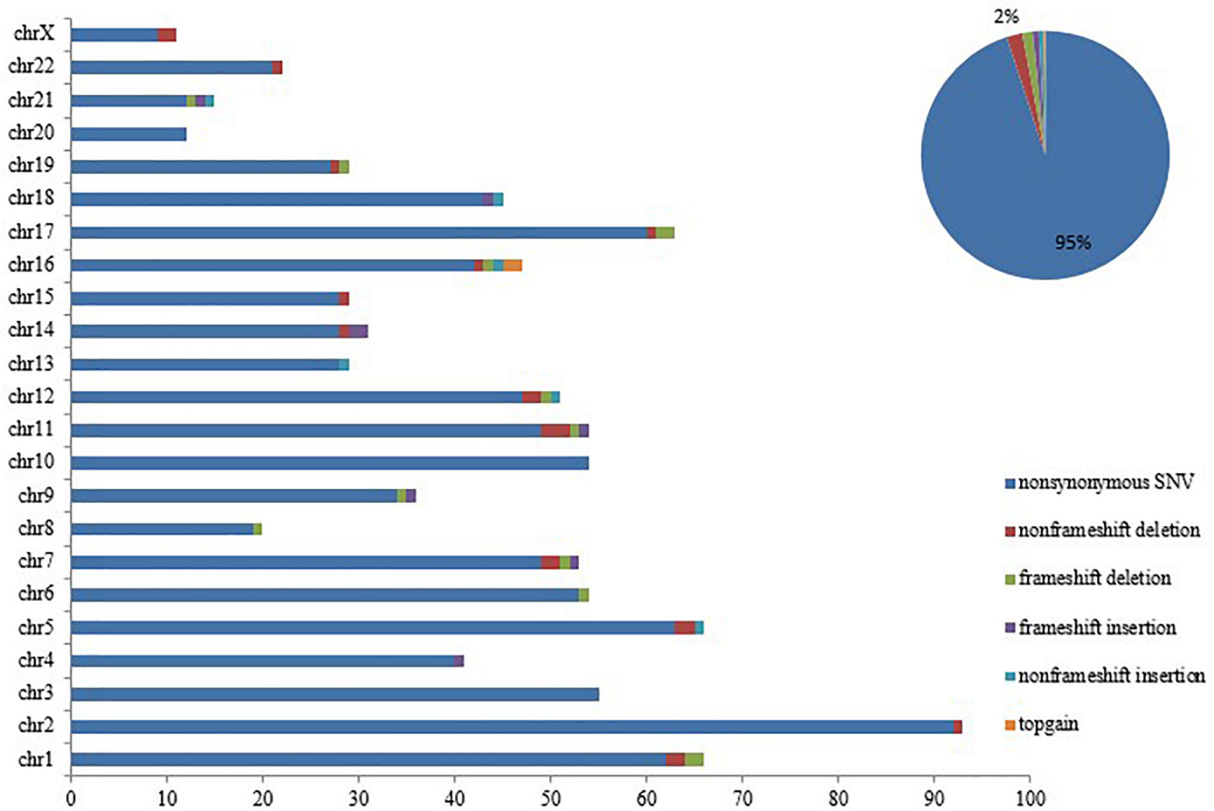

FIGURE 4 | *SETD2* mutation caused genomic instability. Number and type of somatic mutations newly identified in each chromosome when the patient progressed to AML, showing a majority of nonsynonymous variants.

TABLE 4 | Newly somatic variants identified by whole genome sequencing when MDS progression to AML.

Chromo some	Mutation type	Mutation location	AA change	SIFT	Polyphen2 -HDIV	Polyphen2 -HVAR	Frequency
Chr4	Nonsynonymous SNV	*TET2*:NM_001127208:exon3:c.C86G	p.(P29R)	D	D	P	40%
Chr13	Nonsynonymous SNV	*FLT3*:NM_004119:exon9:c.A1073T	p.(D358V)	T	B	B	63%
Chr13	Nonsynonymous SNV	*FLT3*:NM_004119::exon6:c.C680T	p.(T227M)	T	D	P	59%
Chr17	Nonsynonymous SNV	*TP53*:NM_001126115:exon3:c.C326T	p.(S109F)	D	D	D	100%
Chr20	Frameshift deletion	*ASXL1*:NM_015338:exon12:c.2128delG	p.(G710fs)	D	D	D	50%

This finding seemed to be different from previous research. Sun group reported that *SETD2* loss did not affect Wnt/β-catenin signaling in pancreatic ductal adenocarcinoma cells in the context of $Kras^{G12D9}$ (Niu et al., 2020). Yuan group demonstrated that *SETD2* regulated the Wnt pathway indirectly by altering splicing of *DVL2* (Yuan et al., 2017). Given the above research results, we hypothesized that *SETD2* loss can cooperate with other driver mutations to regulate Wnt/β-catenin signaling in the development of MDS. In order to find some clues about the secondary mutations that cause AML transformation, we performed a whole genome sequence of BM cells from the patient at different stages of disease. *ASXL1* and *TET2* mutations were newly detected; non-histone targets of *STED2* also have been found, such as *TP53* and *FLT3* mutations, which implicated a vital role in cell cycle signaling. Despite many additional complicated factors, including the patient receiving decitabine treatment and the discovery of new genetic mutations, it was indicated that *DVL3* was the major isoform among *DVLs* in MDS.

Was there any other mechanism involved to mediate the function of *SETD2* in the transformation from MDS to AML? It was unclear. First, the incidence of *SETD2* gene mutations was low, and we couldn't observe the up-regulation of *DVL3* gene by *SETD2* from other patients. Secondly, due to the scarcity of primary tumor cells, we were not able to further study the *SETD2* gene on epigenetic regulation of RNA processing. Finally, given the evolution of cloned genes, the role of synergistic genes in regulating Wnt signaling pathways couldn't be clearly defined. We only described this phenomenon that *SETD2* modulated Wnt signaling by regulating *DVL3* expression in a patient with MDS. Next, it would be necessary to further verify the regulation

mechanism of *SETD2* on Wnt signaling pathway in *SETD2* gene knockout mouse model.

In summary, we demonstrated that *SETD2* alterations were associated with worse PFS in Chinese patients with MDS, in addition, *SETD2* loss may modulate genomic stability and upregulate *DVL3* expression through Wnt/β-catenin signaling. Although additional studies are needed to elucidate the biological importance of *SETD2* mutations in MDS, our data provided insights into the role of *SETD2* in MDS and suggested that this gene may be a novel therapeutic target in MDS, as well as other human cancers with *SETD2* deficiency.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Ruijin Hospital North, Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL proposed the concept of the study and was involved in the patient's clinical characterization. JL and ZP performed the patient's genetic analysis. YC was involved in all steps of the functional study. JL and FL prepared the manuscript. All authors critically reviewed 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/fgene.2020.00794/full#supplementary-material

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Defined Mathematical Relationships Among Cancer Cells Suggest Modular Growth in Tumor Progression and Highlight Developmental Features Consistent With a Para-Embryonic Nature of Cancer

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Several similarities between the embryo development and the cancer process suggest the para-embryonic nature of tumors. Starting from an initial cancer stem cell (i-CSC) as a para-embryonic stem cell (p-ESC), a hierarchic sequence of CSCs (CSC_1s, CSC_2s, CSC_3s) and non-CSCs [cancer progenitor cells (CPCs), cancer differentiated cells (CDCs)] would be generated, mimicking an ectopic rudimentary ontogenesis. Such a proposed heterogeneous cell hierarchy within the tumor structure would suggest a tumor growth model consistent with experimental data reported for mammary tumors. By tabulating the theoretical data according to this model, it is possible to identify defined mathematical relationships between cancer cells (CSCs and non-CSCs) that are surprisingly similar to experimental data. Moreover, starting from this model, it is possible to speculate that, during progression, tumor growth would occur in a modular way that recalls the propagation of tumor spheres *in vitro*. All these considerations favor a comparison among normal blastocysts (as *in vitro* embryos), initial avascular tumors (as *in vivo* abnormal blastocysts) and tumor spheres (as *in vitro* abnormal blastocysts). In conclusion, this work provides further support for the para-embryonic nature of the cancer process, as recently theorized.

Keywords: tumor propagation, tumor hierarchy, cancer stem cell (CSC), tumor sphere, embryo

INTRODUCTION

It has been theorized recently that several similarities exist between the tumor process and the embryo development (Manzo, 2019). Starting from an initial cancer stem cell (i-CSC/CSC_0), similar to an ESC without genomic homeostasis (para–ESC, p-ESC), after implantation in a niche, primary self-renewing cancer stem cells (CSC_1s) would arise, corresponding to epiblast cells. CSC_1s would then generate secondary proliferating CSCs (CSC_2s), equivalent to hypoblast cells. CSC_1s and CSC_2s, with an epithelial phenotype, would generate, together, tertiary CSCs (CSC_3s)

with a mesenchymal phenotype, corresponding to mesodermal precursors at the primitive streak (PS). Under favorable stereotrophic conditions (normoxia), CSC_3s would undergo asymmetric proliferation and pre-differentiation into cancer progenitor cells (CPCs) and then into cancer differentiated cells (CDCs), thus giving defined cell heterogeneity and hierarchy (Marjanovic et al., 2013; Singh et al., 2015), mimicking an ectopic rudimentary somito-histo-organogenesis process (Reya et al., 2001; Gibbs, 2009; Ma et al., 2010). In contrast, under unfavorable stereotrophic conditions (hypoxia), CSC_3s would delaminate and migrate as quiescent micro-metastases, mimicking morphogenetic movements and localizing in metastatic niches (Cabrera et al., 2015; Singh et al., 2015; Yang et al., 2018). Here, specific signals, similar to those occurring in the gastrula inductions, would induce an EMT/MET switch (Thiery et al., 2009; Liu et al., 2014) reverting quiescent CSC_3s to proliferating CSC_1s. These cells would be able to generate macro-metastases with the same cell hierarchy as their primary tumors (Marjanovic et al., 2013). Now, I intend to show that the above-proposed tumor hierarchy, from CSCs to CDCs, allows the prediction of a tumor proliferation model that is in strong agreement with some experimental data reported for mammary tumors (Liu et al., 2014). Therefore, it is possible to identify specific mathematical relationships among cancer cells (CCs) occurring in the tumor mass. Moreover, this model suggests that during progression tumor growth might occur in a modular way, which recalls features of tumor spheres and pre-implantation blastocysts (Johnson et al., 2013; Vinnitsky, 2014).

CELL HETEROGENEITY, HIERARCHY, AND PLASTICITY IN CANCER

The tumor bulk consists of several types of cells, encompassing Cancer cells (CCs), stroma cells, endothelial cells, and immune cells (Hanahan and Weinberg, 2011). In many tumors, phenotypic and functional heterogeneity among the various cells exists (Marjanovic et al., 2013; Singh et al., 2015), arising from different factors: endogenous, like genetic (mutations) and epigenetic (miRNA, HLA-G, HIF, TGF-beta, BMP); and exogenous, such as niche contact, microenvironment nutrients, pH, space, chemotherapeutic agents. Currently, three different theories try to explain the cell hierarchy and heterogeneity in tumors: (a) the clonal evolution model, (b) the classical CSC model, and (c) the plastic CSC model (Singh et al., 2015). The clonal evolution model proposes that stochastic accumulating mutational events create raw material for the selection of clones of novel cell populations in the same tumor. Each of these cells would be able to generate metastases with particular features, which are different for other metastases and primary tumors. Since it is generally shown that metastases recapitulate the cell hierarchy of the primary tumor in terms of cell type and percentage (Gupta et al., 2011; Marjanovic et al., 2013; Liu et al., 2014; Cabrera et al., 2015; Singh et al., 2015), the clonal model seems unrealistic. The classical CSC model proposes that tumor heterogeneity arises from CSCs that transit through different states (epithelial and mesenchymal) of stemness and differentiation (CPCs and CDCs) by unidirectional conversion from CSCs to non-CSCs (Singh et al., 2015). This model, where CSCs would be at the apex of the process, might better account for heterogeneity and hierarchy of cells in the same tumor, but it does not account for recent reports showing that non-CSCs might revert to CSCs (Chaffer et al., 2011; Gupta et al., 2011; Kim et al., 2013; Singh et al., 2015; Lu et al., 2020). The plastic CSC model proposes that bidirectional conversions are possible between CSCs and non-CSCs, suggesting that during the tumor process, non-CSCs might be induced into CSCs, thus creating new tumor populations (Chaffer et al., 2011; Kim et al., 2013; Singh et al., 2015; Lu et al., 2020). This model might account for both heterogeneity and hierarchy by plasticity of non-CSCs through a context-dependent behavior influenced by microenvironmental signals. Recently, I suggested that tumor heterogeneity and hierarchy might result from the para-embryonic nature of the i-CSC/CSC_0 (Manzo, 2019), which, by the reactivation of an intrinsic genic program, would give rise to a sort of ectopic rudimentary somito-histo-organogenesis, tracing in some way that of the tissue of origin (Reya et al., 2001; Gibbs, 2009; Levings et al., 2009; Ma et al., 2010). Here, naturally epithelial, mesenchymal, progenitor, and differentiated tumor cells would be progressively generated (Bradshaw et al., 2016). Such a genic program would also be realized within related macro-metastases, accounting for the fact that, in general, metastatic cell heterogeneity and hierarchy recapitulate those of the primary tumor. On the other hand, stochastic mutations in the genic program of some CSCs or epigenetic and micro-environmental factors would also be responsible for metastases with a cell heterogeneity different from that in the primary tumor. The plasticity of non-CSCs reverting to a CSC state might be made possible by the genetic instability caused by the absence of genomic homeostasis in the i-CSC/CSC_0 and handed down throughout all its progeny, including CDCs. This instability would allow non-CSCs to be *de novo* reactivated (neo-re-programmed) in their pluripotency gene regulatory network (OCT4, SOX2, NANONG, KLF4, MYC) by endogenous, niche and/or microenvironmental signals, probably in a different way from the original i-CSC/CSC_0, thus generating new tumor cell populations (Iliopulos et al., 2011; Kim et al., 2013; Cabrera et al., 2015; Singh et al., 2015; Yang et al., 2018; Lu et al., 2020). Depending on its genetic, epigenetic and microenvironment conditions, a tumor cell could thus realize a defined genic program ("inductive gene chain") that confers specific phenotypic and physio-pathological features, responsible for a peculiar cell heterogeneity and hierarchy.

THEORETICAL PROLIFERATION MODEL IN CANCER: THE TUMOR GROWTH MODULE

On the basis of the hypothesized p-ESC nature of the i-CSC/CSC_0, I propose the following model for the establishment of cell heterogeneity and hierarchy within the tumor histological structure (**Figure 1**). In a merely theoretical way, considering an i-CSC/CSC_0 and a niche able to contain only (for simplicity) two CSCs, the following events would occur: (a) allocation of

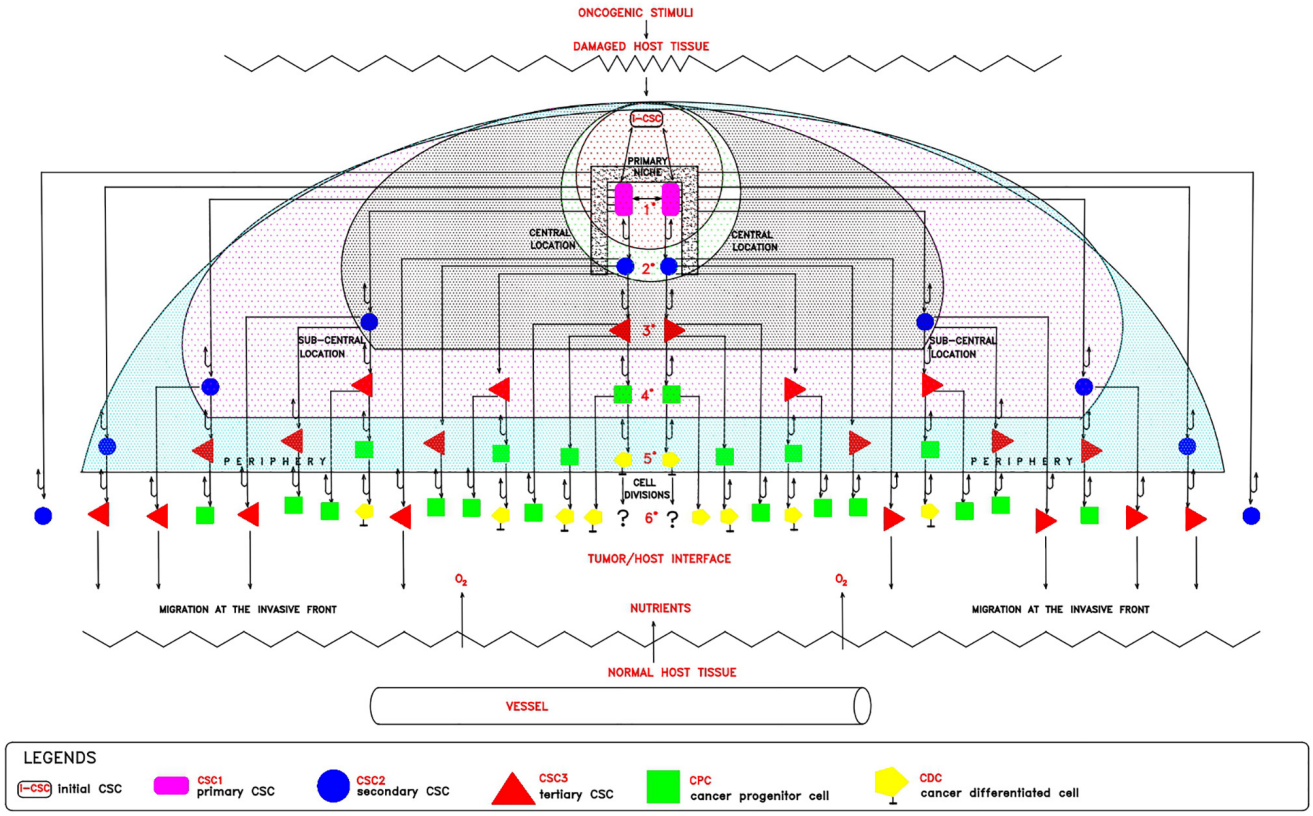

FIGURE 1 | Theoretical tumor growth model: cell hetrogeneity and hierarchy. In a damaged host tissue, the initial cancer stem cell (iCSC/CSC_0, white rectangle) would install itself in a primary niche (black square), where, by expanding symmetrical self-renewal, would generate 2 epithelial CSC_1s (violet rectangles) located at a central position. Each CSC_1, by committing asymmetrical self-renewal, would generate 1 maternal CSC_1, remaining at a central position in the niche, and 1 epithelial committed CSC_2 (blue circles), located at a peri-niche sub-central position. Each CSC_2, by asymmetrical autocrine/paracrine division, would generate 1 maternal CSC_2 and 1 mesenchymal CSC_3 (red triangles), located in a more peripheral position. After a certain division number, CSC_3s generated at early stages, would become more external and proximal to the normoxic host tissue, where they proliferate by asymmetrical division, yielding 1 maternal CSC_3 and 1 pre-differentiated CPC (green squares) located at the border of the process, at the interface between the tumor and the host. CSC_3s generated at later stages, more internal and thus under hypoxic conditions, would remain quiescent and migrate at the invasive front in search of survival conditions in metastatic niches. Each CPC, by asymmetrical division, would yield 1 maternal CPC and 1 differentiated CDC (yellow pentagons). At this point, the question arises as to whether or not CDCs, differentiated but genetically unstable, could further proliferate.

i-CSC/CSC_0 in a niche, at the apex of the entire process, and subsequent CSC_0/CSC_1 transition (Nichols and Smith, 2009); (b) initial expanding symmetrical (Morrison and Kimble, 2006; Norton and Popel, 2014) self-renewal of CSC_1, yielding two epithelial CSC_1s anchored to the niche (Niola et al., 2012); (c) committing asymmetrical (Knoblich, 2008; Pattabiraman and Weimberg, 2014) self-renewal of CSC_1s, each yielding a maternal CSC_1 at a central position in the niche and a committed epithelial daughter CSC_2 in a sub-central position at the niche boundaries (Liu et al., 2014; Norton and Popel, 2014); (d) asymmetrical (Knoblich, 2008; Pattabiraman and Weimberg, 2014) autocrine/paracrine proliferation of CSC_2s, each yielding a maternal epithelial CSC_2 and (via EMT) a mesenchymal daughter CSC_3 in a more peripheral position; (e) quiescence of more internal CSC_3s and their migration externally at the tumor invasive front (Liu et al., 2014; Staneva et al., 2019); (f) asymmetrical (Knoblich, 2008; Norton and Popel, 2014; Pattabiraman and Weimberg, 2014) division of more external CSC_3s, each yielding a maternal CSC_3 and a CPC in a more peripheral position of the process (Liu et al., 2014; Staneva et al., 2019); and (g) asymmetrical (Knoblich, 2008; Pattabiraman and Weimberg, 2014) differentiation division of CPCs, each yielding a maternal CPC and a CDC, at the interface with the host normal tissues. Within this proliferation model, CSC_1s-CSC_2s-CSC_3s-CPCs-CDCs would constitute a defined "tumor growth module." It is possible that such a theoretical proliferation model might account for (1) the various types of CCs present in the bulk of mammary tumors (Liu et al., 2014); (2) the different (epithelial and mesenchymal) CSC phenotypes ($ALDH1^+$ $CD44^+$ $Ki67^+$/hypothetical CSC_1; $ALDH1^+$ $CD44^-$ $Ki67^+$/hypothetical CSC_2; $ALDH1^-$ $CD44^+$ $Ki67^-$/hypothetical CSC_3) detected in mammary tumors (Liu et al., 2014; Manzo, 2019); (3) the hierarchy of the various CSCs and non-CSCs present in a tumor (Liu et al., 2014); (4) the histological tumor structure, where CSCs would naturally remain internal, surrounded by more differentiated tumor cells (Liu et al., 2014;

Singh et al., 2015); and (5) the position of CSC_3s generated at early divisions, which would become progressively more external and proximal to the normoxic host tissues, where favorable micro-environmental conditions (space, oxygen, nutrients, pH) exist (**Figure 1**). Here, they could undergo EMT/MET switch, subsequent asymmetric division and differentiation in CPCs and then in CDCs, thus generating a growth module with a defined cell hierarchy, responsible for a peripheral finger-like morphology (Norton and Popel, 2014). With regards to the CDCs, the question arises of whether or not they are still proliferating: in general, proliferation and differentiation are mutually exclusive, as also it occurs in CCs (Ruijtenberg and van den Heuvel, 2016). However, coincident occurrence of cell division and a differentiated state have also been reported in CCs (Sage et al., 2005; Ajioka et al., 2007); moreover, the eventual occurrence of dividing pre-differentiated CCs must be considered. In contrast, CSC_3s generated at later divisions would remain more internal and thus under unfavorable hypoxic conditions. Consequently, in an attempt to survive, they would migrate externally in spatially coordinated migration patterns (Thiery et al., 2009; Tiwari et al., 2012; Staneva et al., 2019), at the interface with normal vascularized host tissues, where better stereo-trophic conditions exist, thus creating an invasive front. Here, they could install in metastatic niches as dormant CSC_3s by EMT signals (WNT, TGFb) and eventually revert to self-renewing CSC_1s by MET signals (BMP, LIF) (Thiery et al., 2009; Liu et al., 2014; Grosse-Wilde et al., 2015).

MODULAR GROWTH IN AVASCULAR TUMORS

The proposed proliferation model would generate tumor growth modules (CSC_1-CSC_2-CSC_3-CPC-CDC) that might be at the basis of and account for the structure and features of the avascular tumor bulk. In particular, mesenchymal CSC_3s generated early in a tumor growth module would lie in favorable stereotrophic conditions, so they could proliferate, yielding a progeny of CPCs and then CDCs. This progeny could form a hierarchic histological structure that might appear as growth-cordfingers (Norton and Popel, 2014; **Figure 2**). On the other hand, mesenchymal CSC_3s generated later within a growth module would lie in unfavorable stereotrophic conditions, so they would be induced to migrate externally for survival. If they find a

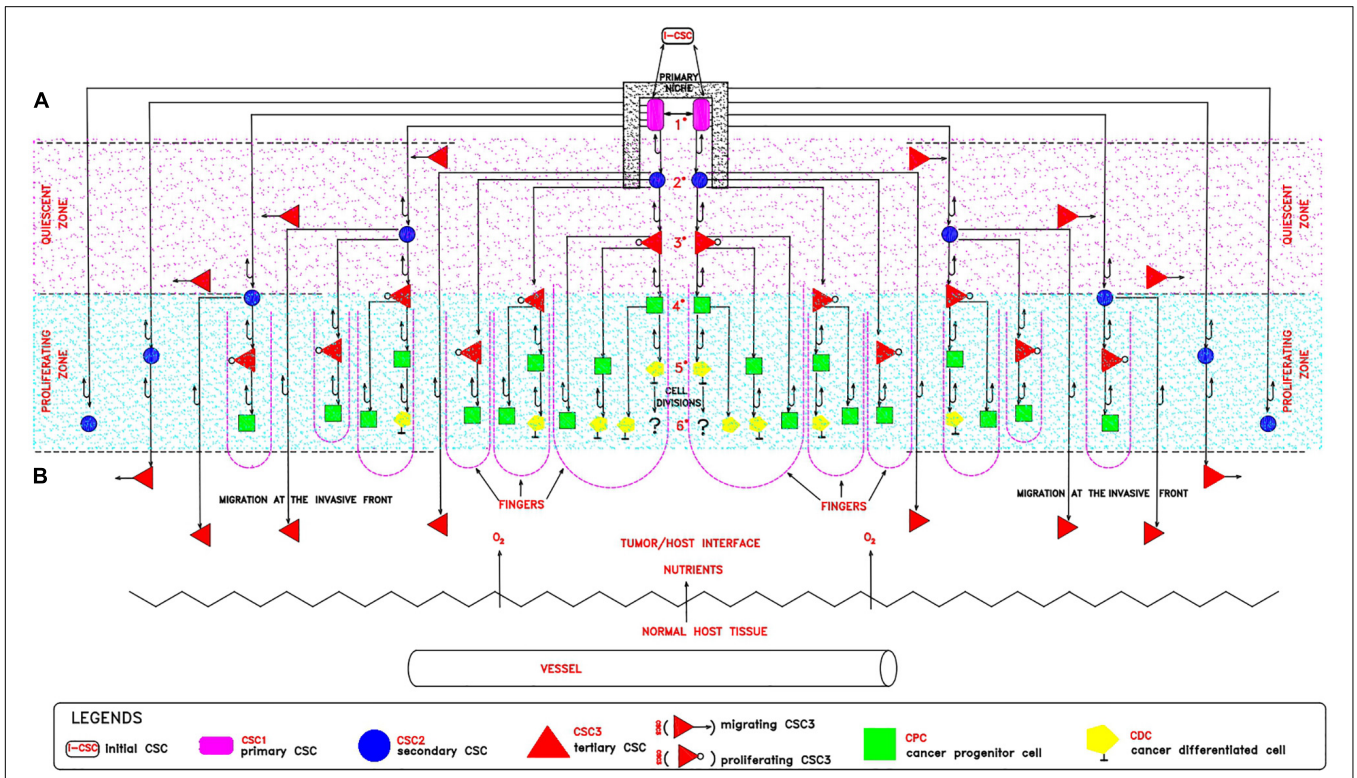

FIGURE 2 | Theoretical tumor growth module: a cord-finger structure. In a tumor growth module, it is possible to distinguish: **(A)** An internal hypoxic zone (pink color) where quiescent CSC_3s lie, generated at later divisions and migrating externally in spatially coordinated patterns, toward the tumor/host interface, endowed with more favorable stereo-trophic conditions, thus creating an invasive front. Here, they could install in metastatic niches as dormant CSC_3s by EMT environmental signals (WNT, TGFb) and eventually revert to self-renewing CSC_1s by MET signals (BMP, LIF). **(B)** An external normoxic zone (light-blue color), where CSC_3s lie, generated at earlier divisions and thus more proximal to the tumor/host interface, where favorable micro-environmental conditions (space, oxygen, nutrients, pH) exist. Here, these cells could undergo EMT/MET switch, subsequent asymmetric division and differentiation in CPCs and then in CDCs, thus generating a growth module with a defined cell hierarchy, responsible for a peripheral finger-like morphology (violet broken lines). With regards to the CDCs, the question arises of whether or not they are still proliferating.

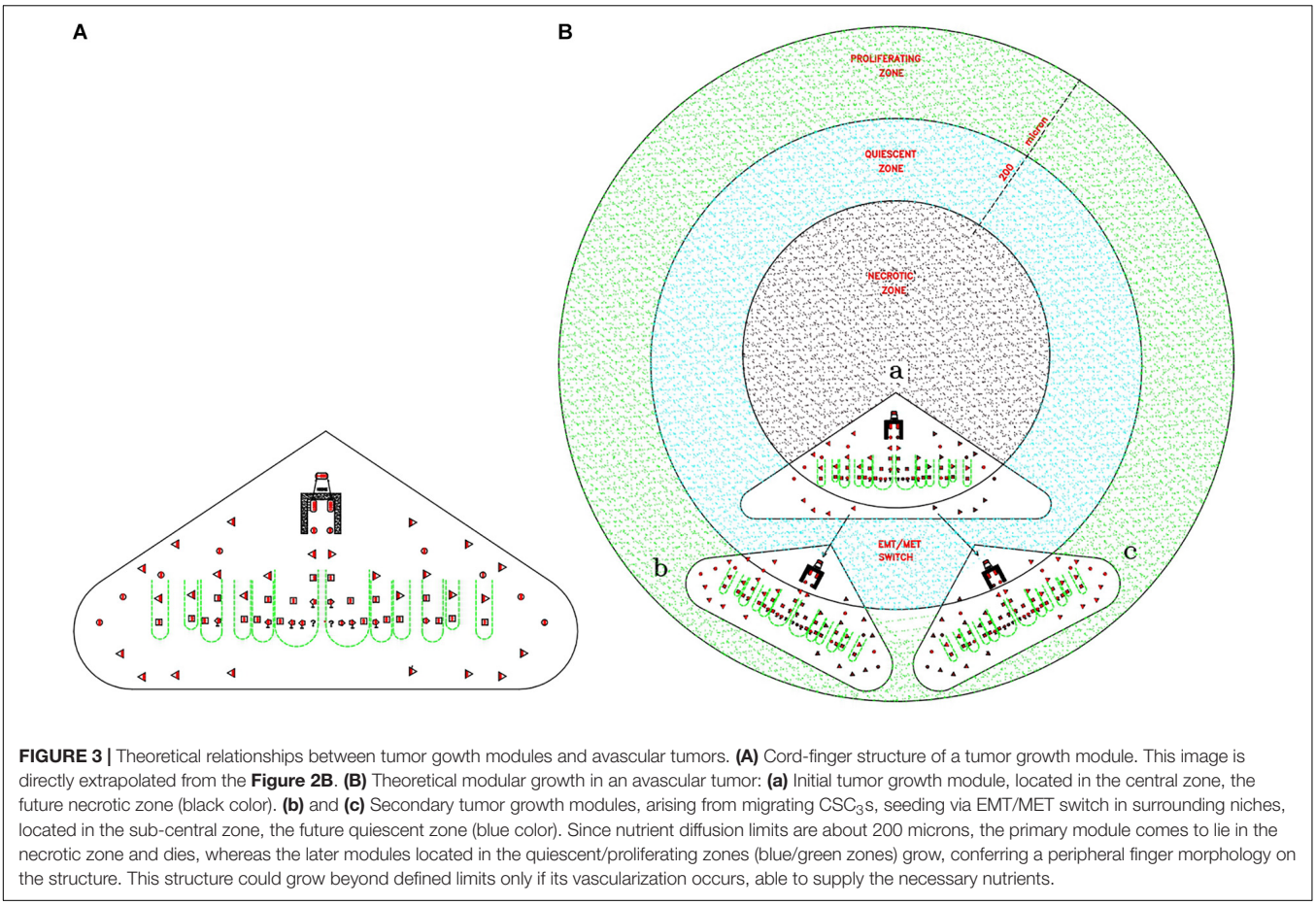

FIGURE 3 | Theoretical relationships between tumor gowth modules and avascular tumors. **(A)** Cord-finger structure of a tumor growth module. This image is directly extrapolated from the **Figure 2B**. **(B)** Theoretical modular growth in an avascular tumor: **(a)** Initial tumor growth module, located in the central zone, the future necrotic zone (black color). **(b)** and **(c)** Secondary tumor growth modules, arising from migrating CSC_3s, seeding via EMT/MET switch in surrounding niches, located in the sub-central zone, the future quiescent zone (blue color). Since nutrient diffusion limits are about 200 microns, the primary module comes to lie in the necrotic zone and dies, whereas the later modules located in the quiescent/proliferating zones (blue/green zones) grow, conferring a peripheral finger morphology on the structure. This structure could grow beyond defined limits only if its vascularization occurs, able to supply the necessary nutrients.

new niche, they would self-seed (Norton and Popel, 2014) and, by specific signals, undergo EMT/MET switch, becoming self-renewing CSC_1s able to generate new tumor modules. In such a way, tumor growth could occur by reiterated production of defined cell modules, generating a spherical avascular mass. This might expand until it reaches a diameter of approximately 400 microns, since diffusion and the supply of nutrients and oxygen at the core cells is not possible beyond about 200 microns (Hamilton and Rath, 2019). Assuming for the module cells a middle diameter of about 15 microns, this fact would imply that an avascular tumor bulk might contain about 13 tumor cell layers. Beyond this limit, tumor avascular growth could occur only externally with a simultaneous death of core cells. In such a way, an advanced avascular tumor mass could be a sphere made of (a) an anoxic central zone with necrotic tumor cells, presumably the earlier tumor modules; (b) a sub-central hypoxic zone with the later generated quiescent CSC_3s that try to migrate externally (Staneva et al., 2019) in search of niches to self-seed around or for metastasizing elsewhere (Norton and Popel, 2014); and (c) a peripheral normoxic zone with the earlier generated proliferating CSC_3s and their numerous progeny of CPCs and CDCs, resulting together in a cord-finger morphology (Norton and Popel, 2014; **Figures 2**, **3**). Thus, this tumor proliferation model would generate structures that appear to be very similar to real initial avascular tumors and multicellular tumor spheroids (MCTS) (Millard et al., 2017; Hamilton and Rath, 2019; Scientific Reports and Nature Research, 2019; **Figure 4**).

MATHEMATICAL RELATIONSHIPS AMONG CANCER CELLS

In the model proposed in **Figure 1** it is possible to detect numerical relationships among all the CC typologies in a tumor, which surprisingly agree with experimental data shown in a study on 45 primary breast tumors (Liu et al., 2014). By tabulating the theoretical data proposed in **Figure 1**, it is possible to find well-defined mathematical relationships between CSCs (CSC_1s, CSC_2s, CSC_3s) and non-CSCs (CPCs, CDCs) at each (n) cell division. Starting from a hypothetical low and stable number (two, for simplicity) of CSC_1s in a niche, for each (n) division, it is possible to define the following relationships (**Table 1**):

$$(CSC_1s)_n = 2 \quad (CSC_2s)_n = (CSC_1s + CSC_2s)_{n-1}$$
$$(CSC_3s)_n = (CSC_2s + CSC_3s)_{n-1}$$
$$(CPCs)_n = (CSC_3s + CPCs)_{n-1}$$
$$(CDCs)_n = (CPCs + CDCs)_{n-1}$$

These relationships theoretically allow us to know, at each (n) division, a presumed total CC number as a sum of the number

TABLE 1 | Theoretical mathematical relationships among cancer cells in tumors.

	CSCs			Non – CSCs		Total CSCs		CSCs/CCs Presumed ratio
a i-CSC Division number (n)	b (CSCs1)$_n$ (constant)	c (CSCs2)$_n$ (CSCs1+CSCs2)$_{n-1}$	d (CSCs3)$_n$ (CSCs2+CSCs3)$_{n-1}$	e (CPCs)$_n$ (CSCs3+CPCs)$_{n-1}$	f (CDCs)$_n$ (CPCs+CDCs)$_{n-1}$	g (mCDCs)$_n$ (CDCs + 2mCDCs)$_{n-1}$	h Theoretic i CCs (2^n)	Presumed CCs (2^n-mCDCs)	j**
1	2	0	0	0	0	0	2	2	2/2 = 1
2	2	2	0	0	0	0	4	4	4/4 = 1
3	2 → + ←	4	2	0	0	0	8	8	8/8 = 1
4	2 | →	6 → + ←	6	2	0	0	16	16	14/16 = 0.87
5	2	8 | →	12 → + ←	8	2	0	32	32	22/32 = 0.69
6*	2	10	20 | →	20 → + ←	10	2	64	62	32/62 = 0.52
7	2	12	30	40 | →	30 → + ←2×	14	128	114	44/128 = 0.34
8	2	14	42	70	70 | →	58	256	198	58/198 = 0.29
9	2 (0.61 %)	16 (4.91 %)	56 (17.18 %)	112	140	186	512	326	74/326 = 0.23
10***	2 (0.39 %)	18 (3.51 %)	72 (14.06 %)	168	252	512	1024	512	92/512 = 0.18
11	2 (0.26 %)	20 (2.59 %)	90 (11.66 %)	240	420	1276	2048	772	112/772 = 0.14
12	2	22	110	330	660	2972	4096	1124	134/1124 = 0.12
13	…	…	…	…	…	…	…	…	

Tabular representation of what is proposed in ***Figure 1****. Theoretical mathematical relationships among CCs in a tumor process could be obtained for each cell typology (CSCs and non-CSCs), as reported in the formulae at the top of* ***Table 1****. These formulae can be obtained following the sequence of arrows, as shown. Regarding the total number of CCs, a discrepancy between theoretical (column h) and presumed (column i) values is the result of missing CDCs (column g) arising at the sixth division (*) and increasing progressively in subsequent divisions. The mCDCs might be a natural occurrence if CDCs cannot proliferate, whereas they might be the result of apoptotic death if CDCs are still proliferating; however, in this case, it would not be explainable how a mathematical model might foresee it. The data in* ***Table 1*** *allow us to determine the presumed ratio between CSCs and total CCs (**), progressively decreasing during tumor progression, as experimentally detected in mammary tumors. Note the high convergence between presumed and experimental CSC percentages (about 18%) at the n = 10 division number (***).*

of each cell typology. In particular, it might be noted that (a) for values of (n) from 0 to 5, the total CC number is expressed as a numerical doubling (2^n) (**Table 1h,i**) (b) from a value (n) of 6, the total CC number decreases progressively compared with (2^n) (**Table 1h,i**), because of missing CDCs (mCDCs), in a quantity expressed by the relationship (**Table 1g**):

$$(\mathrm{mCDCs})_n = (\mathrm{CDCs} + 2 \times \mathrm{mCDCs})_{n-1}$$

(c) at a value (n) of 10, the presumed CC number is exactly half of (2^n), at a value (n) of 11, about one third, and at a value (n) of 12, a little more than one fourth (**Table 1h,i**). Thus, the total CC number appears to become progressively more self-limiting; nevertheless, the CSC quantity decreases gradually compared with the total CCs, according to the experimental ratio reported in the literature (**Table 1j**).

(d) At a value (n) of 10, the percentages of CSC_1s, CSC_2s, and CSC_3s result, respectively, in 0.39, 3.51, and 14.06%, with a total of 17.96% CSCs. Surprisingly, these theoretical data, concerning a single niche, are strongly similar to the experimental data found in the mammary tumor mass, namely: 0.084% for $ALDH1^+$ $CD44^+$ $Ki67^+$ CSCs (hypothetical CSC_1s); 5.54% for $ALDH1^+$ $CD44^-$ $Ki67^+$ CSCs (hypothetical CSC_2s); 12.87% for $ALDH1^-$ $CD44^+$ $Ki67^-$ CSCs (hypothetical CSC_3s); and 18.494% for total CSCs (Liu et al., 2014). These similar (presumed/experimental) percentages (about 18%) also occur starting from a niche with a different (3, 4,. . .) initial CSC_1 number. Inside these percentages, the discrepancy for CSC1s (0.39 to 0.084%, about 5 to 1) and CSC2s (3.51 to 5.54%, about 1 to 2) might be due to the fact that the proliferation rate for a single theoretical niche is assumed as defined, while in the tumor bulk many niches could have asynchronous growth and a variable proliferation rate. Moreover, in a computational model, stem cell percentages have been found to be between 0.2 and 15%, depending on the simulation parameters (Norton and Popel, 2014). These similarities might thus indicate a true correspondence between $ALDH1^+$ $CD44^+$ $Ki67^+$CSCs and CSC_1s, $ALDH1^+$ $CD44^-$ $Ki67^+$CSCs and CSC_2s, $ALDH1^-$ $CD44^+$ $Ki67^-$ CSCs and CSC_3s, and, consequently, a possible real existence of the hypothesized CSC_1s, CSC_2s, and CSC_3s (Manzo, 2019).

SIMILARITIES AMONG AVASCULAR TUMORS, TUMOR SPHERES, AND BLASTOCYSTS

Notably, at (n) = 10, many important events seem to occur, as described above. At (n) > 10, the correspondence (about 18%) between presumed (17.96%) and experimental (18.494%) CSCs for a single niche tends to diminish progressively. Since the experimental data refer to tumor bulks, certainly with more than 10 cell divisions, it would be possible to question how this correspondence might be conserved in the tumor mass. I therefore hypothesize that it might occur through the proposed "modular growth," which is able to maintain these percentages throughout tumor progression. In particular,

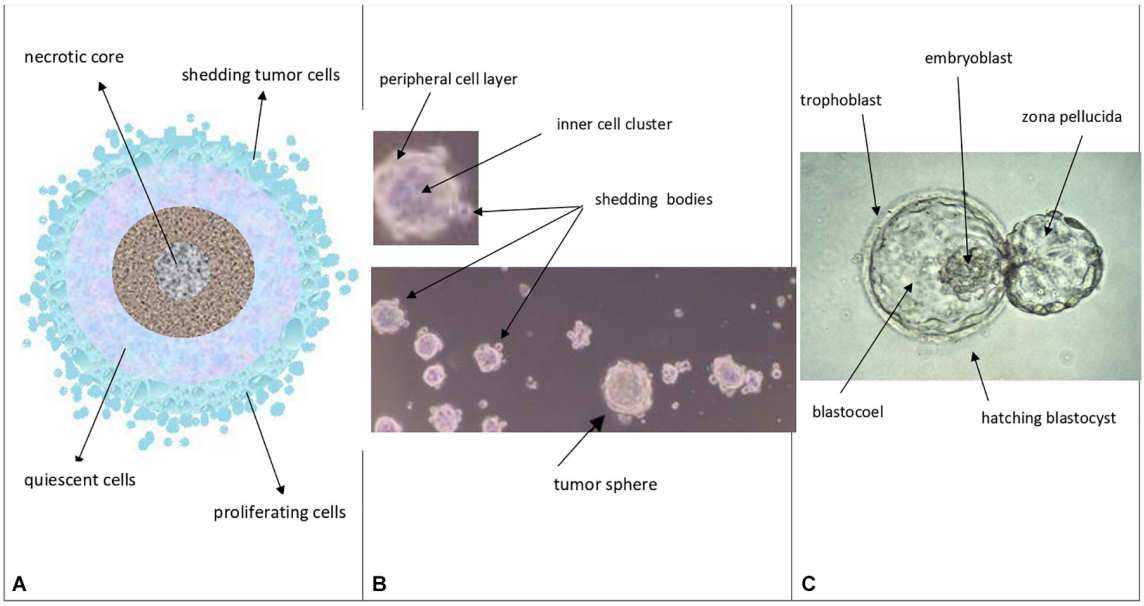

FIGURE 4 | Theoretical comparison among avascular tumors **(A)**, tumor spheres **(B)**, and normal blastocysts **(C)** A. Avascular tumors (as *in vivo* abnormal blastocysts): external proliferating cell layers (light-blue color), middle quiescent cells (gray color) and necrotic core cells (dark colors) are depicted. B. Tumor spheres (as *in vitro* abnormal blastocysts): a layered cell distribution, like in avascular tumors, a peripheral cell layer, similar to the blastocyst trophectoderm, and an inner cell cluster, similar to the blastocyst ICM can be observed; shedding bodies from the sphere surfaces can also be noted (modified and adapted from Bond et al., Plos One. 2013). C. Normal hatching blastocysts (as *in vitro* embryos): trophectoderm, ICM (embryoblast) and blastocoel cavity are indicated, together to the "zona pellucida" (modified from: Human blastocyst hatching. Credit: K. Hardy. CC BY).

TABLE 2 | Similar features among avascular tumors, tumor spheres and preimplantation blastocysts.

Similar features	Avascular tumors	Tumor spheres	Preimplantation blastocysts
Presumed nature	In vivo abnormal blastocysts	In vitro abnormal blastocysts	In vitro normal embryos
Form	Spherical	Spherical	Spherical
Dimension (microns)	400 (about)	50–250 (and more)	200 (and more)
Cell distribution	Layered	Layered	Layered
Cell types	CSC_S-CPC-CDC	CSC_S-CPC-CDC	Trophoblast cells
			Embryoblast cells (ESC_S)
Molecular markers	OCT4-SOX2-NANOG-CD44-ALDH1	OCT4-SOX2-NANOG-CD44-ALDH1	OCT4-SOX2-NANOG-CD44-ALDH1
Structure:			
outer layers	Proliferating cells	Proliferating cells	Proliferating trophoblasts
middle layers	Quiescient cells	Quiescient cells	Inner cells
core	Necrotic cells	Necrotic cells	Apoptotic cells
			Blastocoel
Shedding structures	Tumor cells and fragments	Shedding bodies	Hatching blastocyst/zona pellucida

The major similarities among avascular tumors, tumor spheres and preimplantation blastocysts are summarized and pointed out. This table can also supply some information related to the ***Figure 4****.*

this might be possible if, as proposed earlier, CSC_1s-CSC_2s-CSC_3s-CPCs-CDCs together constituted a tumor growth module (Manzo, 2019; **Figure 2**). This would self-generate after about 10 division cycles, when the cell number would become presumably too large to survive under unfavorable stereotrophic conditions (Hamilton and Rath, 2019). For this reason, some CSC_3s would be induced to delaminate, migrate, and localize in new local or distant niches, where, after EMT/MET switch, they would revert to CSC_1s (O'Brien et al., 2012; Liu et al., 2014; Grosse-Wilde et al., 2015; Yang et al., 2018; Manzo, 2019) and repeat the modular growth process, thus generating structures with a defined cell heterogeneity and hierarchy (Knoblich, 2008; Johnson et al., 2013; Vinnitsky, 2014). A modular growth process appears to occur also when CSCs cultured *in vitro* under defined conditions form solid, round cellular structures with a diameter of about 50–250 microns, named tumor spheres, through joining of smaller aggregates (spheroids), similar to -single tumor modules (Hamilton and Rath, 2019). Spheroids are also found *in vivo*, as circulating tumor clusters, in ascitic fluid of ovarian cancer and pleural

effusions of lung cancers, arising by collective detachment from the tumor bulk (Hamilton and Rath, 2019). These spheroids have a smaller size without the hypoxia and necrotic regions observed in larger 3D structures (Hamilton and Rath, 2019). Tumor spheres are enriched in CSCs, but they also contain non-CSCs, less or more differentiated (CPCs, CDCs) (Cao et al., 2011; Johnson et al., 2013; Liu et al., 2013). The CSCs are endowed with persistent self-renewal, stemness gene expression, high invasiveness, increased tumorigenic potential, and chemo-resistance (Cao et al., 2011; Liu et al., 2013). In such CSCs, expression of NANOG, OCT4, and SOX2 is present, as well as that of ALDH1 and KLF4 (epithelial markers) and CD44 (mesenchymal marker) (Liu et al., 2013). Tumor spheres are tridimensional and mimic the micro-environmental conditions and growth of real tumors. Tumor-sphere cultivation is widely used to analyze the self-renewal capability of CSCs and to enrich these cells from bulk CCs, thus providing a reliable platform for screening potential anti-CSC agents (Knoblich, 2008; Nunes et al., 2018). Large spheroids (400–500 microns in diameter) display a layered cell distribution, also observed in solid avascular tumors: the outer layers are enriched with highly proliferating cells, the middle zone exhibits quiescent cells, and the core contains necrotic cells and acellular regions with hypoxia and nutrient depletion (Norton and Popel, 2014; Galateanu et al., 2016; Millard et al., 2017). Very large tumor spheroids can reach 650 microns in diameter (Zanoni et al., 2016). Morphologically, tumor spheres appear to be defined by a cell layer that resembles the trophectoderm in blastocysts and a cluster of inner cells that resembles the ICM, just like in a preimplantation blastocyst (Johnson et al., 2013; Vinnitsky, 2014) (**Figures 4B,C**). I suggest that tumor spheres could be an artificial condition mimicking *in vitro* the natural conditions of normal pre-implantation blastocysts (Cao et al., 2011; Vinnitsky, 2014; Nunes et al., 2018), as well as those of *in vivo* avascular tumors (**Figures 4A–C** and **Table 2**). Thus, I hypothesize that tumor spheres might be a sort of artificial rudimentary (abnormal) blastocysts which, cultured *in vitro* onto ultralow attachment surfaces in the absence of implantation conditions, display a modular growth behavior similar to that of avascular tumors *in vivo* (Vinnitsky, 2014). This modular growth would also be confirmed by the images of small "shedding" structures, similar to single tumor modules, recently shown on the tumor-sphere surface and released in the surrounding micro-environment (Johnson et al., 2013; Hamilton and Rath, 2019; **Figure 4B**). The release of such structures resembles and could reflect in some way the "hatching" phenomenon of expanded pre-implantation blastocysts, by which these emerge from the zona pellucida to acquire a condition fit for subsequent implantation (Hardy et al., 1989; **Figure 4C**). Larger tumor spheres could maintain their state by inducing the release of cells exceeding a cell number (250–280) which would be optimal for eventual implantation. In the absence of micro-environmental conditions that favor implantation, normal blastocysts in the uterus die, while tumor spheres with defined *in vitro* conditions survive and spread, producing shedding growth modules. These would be presumably similar to *in vivo* initial avascular tumors (Vinnitsky, 2014), which could survive as such (dormant) in the absence of suitable implantation conditions or progress in the presence of such conditions. Multicellular tumor spheroid models closely mimic small avascular tumors *in vivo*, with the presence of proliferative cells (about 40%) surrounding quiescent cells and a necrotic core, and with similar gradients of oxygen, pH, and nutrients (Millard et al., 2017; Hamilton and Rath, 2019) (**Figure 4B**). It has been proposed that tumor spheres fulfill the precondition for a protected niche for dormant tumor cells as an hypoxic niche protected by the outer layers, which exhibit continuous shedding of tumor cells and fragments (Johnson et al., 2013; Hamilton and Rath, 2019).

DISCUSSION AND CONCLUSION

The hypothesis that oncogenesis might be a sort of ectopic rudimentary ontogenesis (Manzo, 2019) would permit us to formulate some considerations and potential explanations for several phenomena: a-Tumor cell heterogeneity and hierarchy, similar in primary and metastatic tumors, might be a natural consequence of the developmental genic program of a de-re-programmed i-CSC/CSC_0 endowed with para-embryonic features (p-ESC) (Manzo, 2019). b-The plasticity of non-CSCs in the CSC conversion might be made possible by the early genomic instability of the i-CSC/CSC_0, handed down throughout all its progeny. Therefore, thanks to this condition, a non-CSC could be newly re-programmed in CSC by intrinsic and/or extrinsic signals, eventually also in a different way to the original i-CSC, thus potentially giving rise to a new tumor cell population, with new co-existent heterogeneity and hierarchy arising in the same primary tumor. c-The proposed cell hierarchy model (**Figure 1**) might account for the global tumor structure shown in mammary tumors (Liu et al., 2014), namely the distinction between CSCs and non-CSCs, the different detected CSC phenotypes, the reciprocal allocation of the different CSCs in the tumor mass, the internal position of CSCs to the external position of the non-CSCs. d-Tabulation of the above proposed cell hierarchy model (**Figure 1**) permits the elaboration of well-defined formulae for calculating the presumed number of each CC typology and, consequently, the presumed total number of CCs and the CSCs/CCs ratio after (*n*) cell division (**Table 1j**). This presumed ratio clearly appears to decrease progressively, in agreement with the experimental data reported in the literature. e-However, the presumed total number of CCs seems to be self-limiting for the occurrence of mCDCs. mCDCs could be the result of a lack of further proliferation of CDCs; but, if CDCs were still proliferating, this fact could be due to a natural apoptotic cell death, similarly to what occurs in embryos throughout ontogenesis (Hardy et al., 1989) and in multicellular spheroids (Nunes et al., 2018). In the embryo, widespread cell death by apoptosis in both TE and ICM normally occurs, increasing substantially by about day 7 (Hardy et al., 1989), namely from the 6/7° "one per day" division. Surprisingly, in **Table 1**, the onset of mCDC occurred just by the 6°cell division and then increased progressively. f-The major indication, resulting from **Table 1**, is the surprising similarity between the presumed and experimental percentage values for CSCs ($ALDH1^+$ $CD44^+$

$Ki67^+/CSC_1$; $ALDH1^+$ $CD44^-$ $Ki67^+/CSC_2$, $ALDH1^-$ $CD44^+$ $Ki67^-/CSC_3$), totaling approximately 18% for mammary tumors. g-Such a quantitative correspondence (about 18%) for CSCs could not be a simple coincidence and, if so, constitute a strong indication for the real existence of CSC_1s, CSC_2s, and CSC_3s (Liu et al., 2014; Manzo, 2019). h-CSC1-CSC_2s-CSC_3s-CPCs-CDCs, together, could constitute a real tumor progression module that determines modular growth able to maintain a substantially constant ratio of about 18% for CSCs in the tumor mass, as detected in mammary tumors (Liu et al., 2014). In conclusion, I believe this work might contain and supply further indications sustaining the para-embryonic nature of the cancer process, as recently theorized (Manzo, 2019).

AUTHOR CONTRIBUTIONS

GM conceived the theory and wrote the manuscript.

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REVIEW
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Regulation of N6-Methyladenosine in the Differentiation of Cancer Stem Cells and Their Fate

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N6-methyladenosine (m6A) is one of the most common internal RNA modifications in eukaryotes. It is a dynamic and reversible process that requires an orchestrated participation of methyltransferase, demethylase, and methylated binding protein. m6A modification can affect RNA degradation, translation, and microRNA processing. m6A plays an important role in the regulation of various processes in living organisms. In addition to being involved in normal physiological processes such as sperm development, immunity, fat differentiation, cell development, and differentiation, it is also involved in tumor progression and stem cell differentiation. Curiously enough, cancer stem cells, a rare group of cells present in malignant tumors, retain the characteristics of stem cells and play an important role in the survival, proliferation, metastasis, and recurrence of cancers. Recently, studies demonstrated that m6A participates in the self-renewal and pluripotent regulation of these stem cells. However, considering that multiple targets of m6A are involved in different physiological processes, the exact role of m6A in cancer progression remains controversial. This article focuses on the mechanism of m6A and its effects on the differentiation of cancer stem cells, to provide a basis for elucidating the tumorigenesis mechanisms and exploring new potential therapeutic approaches.

Keywords: m6A, stem cell, differentiation, cancer, hematopoietic

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INTRODUCTION

N6-methyladenosine (m6A) is one of the most common internal modifications in eukaryotic mRNAs and non-coding RNAs including long non-coding RNAs (lncRNA), microRNAs (miRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and transfer RNAs (tRNAs) (Bokar et al., 1997; Fu et al., 2014). This dynamic and reversible modification was first discovered in the 1970s, and it involves three types of molecules: methyltransferases, demethylases, and methylated binding proteins (Bokar et al., 1997; Fu et al., 2014). Recent emerging studies suggested that m6A is not only involved in the normal physiological processes but also associated with the occurrence of and development of multiple cancers (Deng et al., 2018; Lai et al., 2018; Zhang et al., 2018; Wei et al., 2019).

Stem cells including totipotent stem cells (TSCs), pluripotent stem cells (PSCs), and unipotent stem cells (USCs) have a strong ability of self-renewal, proliferation, and differentiation (Bozdag et al., 2018; Sotthibundhu et al., 2018). TSCs can be differentiated into full organisms, the same way as embryonic stem cells (ESCs) do (Bozdag et al., 2018). PSCs, which are also called mesenchymal stem cells (MSCs), have the potential to differentiate either into a variety of cellular tissues or into different cells of a certain tissue type, like hematopoietic stem cells (HSCs) (Kashima et al., 2018). USCs can only differentiate into one or two closely related cell types, such as the mammary stem cells (Lilja et al., 2018). The stemness of stem cells is determined by the presence of certain protein molecules, and the expression of these molecules is mainly controlled through DNA methylation, histone acetylation, and miRNAs (Moussaieff et al., 2015; Shim and Nam, 2016; Ran et al., 2017; Wang et al., 2017). Based on the rapid development of research strategies and technologies, many stem cells core pluripotency factors have been identified, including Octamer-binding transcription factor 4 (OCT4), SRY-box 2 (SOX2), and NANOG (Hu et al., 2008; Leis et al., 2012; Iv Santaliz-Ruiz et al., 2014). It has been demonstrated that m6A methylation is indispensable for the pluripotency and differentiation of ESCs and HSCs (Batista et al., 2014; Wang et al., 2014; Vu et al., 2017; Li et al., 2018; Weng et al., 2018). These biological properties of stem cells make them a research hotspot, whether in basic scientific research or in clinical medicine research.

In malignant tumors, it has been suggested that some cancer cells such as cancer stem cells (CSCs) have similar biological characteristics as those of stem cells, such as self-renewal ability and multiple differentiation potential, thereby producing heterogeneous tumor cells (Reya et al., 2001; Prasetyanti and Medema, 2017). In 1994, through specific cell surface markers, Lapidot et al. isolated a type of cell with self-renewal and maintenance of malignancy properties from leukemia cells, named as acute myelogenous leukemia stem cells (LSCs); this was the first confirmation of the existence of CSCs (Lapidot et al., 1994). Currently, with infinite proliferation abilities, the important role of CSCs in the occurrence and development of malignant tumors, such as tumor survival, proliferation, metastasis, and recurrence, was confirmed (Reya et al., 2001; Chang, 2016; Pan et al., 2018). Identification and elimination of CSCs in malignant tumors have become a new strategy for treatment. The differentiation of CSCs is controlled by many factors such as abnormal activation of the PI3K/Akt/mTOR axis, Wnt and Notch signaling pathways, and adhesion molecules such as cadherin and integrin that mediate the anchoring of stem cells to their niche (Lin, 2002; Indrayani, 2018; Venkatesh et al., 2018). Recent studies demonstrated that m6A participates in the self-renewal and pluripotent regulation of CSCs (Zhang C. et al., 2016, 2017; Zhang S. et al., 2017). However, as the multiple targets of m6A are involved in different physiological processes, the role of m6A remains controversial. Therefore, this review focuses on the mechanism of m6A and its role in the differentiation of stem cells and CSCs to determine their roles in malignant tumors.

M6A PROCESSES AND THEIR FUNCTIONS

The m6A modification is catalyzed by an unidentified methyltransferase complex containing at least one subunit, METTL3. In some cases, it can be read and erased by reader proteins and demethylases (Roundtree et al., 2017; **Figure 1**). Increasing evidence suggests that m6A modification is misregulated in human cancers and may be ideal targets of cancer therapy (Barbieri and Kouzarides, 2020). The m6A modification affects the pathogenesis of multiple diseases and cancers, not only by affecting coding RNAs but also by affecting non-coding RNAs, such as microRNAs, lncRNAs and circRNAs (Fazi and Fatica, 2019; Zhang et al., 2020).

Methyltransferases

Methyltransferases function as enzymes that act downstream of mRNA adenylate undergoing m6A modification, including methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), Wilms tumor 1-associating protein (WTAP), and KIAA1492 (Schwartz et al., 2014). Previously, it was thought that METTL3 and METTL14 are methyltransferases involving the formation of a heterodimer complex that functions in cellular m6A deposition on mammalian nuclear RNAs (Liu et al., 2014). Recently, it was found that METTL3 plays the role of the main enzyme of methyltransferases, whereas METTL14 promotes the binding of METTL3 to the targeted RNA (Wang et al., 2016). WTAP is responsible for recruiting the METTL3-METTL14 heterodimer complex into nuclear speckles (Wu et al., 2018). WTAP and METTL3-METTL14 are co-localized in the nuclear speckles where they participate in the process of RNA splicing (Liu et al., 2014). Further, it was reported that KIAA1492 is another core protein belonging to methyltransferases, which is also localized in the nuclear speckles; however, its function is unclear (Schwartz et al., 2014). Importantly, the above-mentioned methyltransferases do not work in isolation but rather form a complex in which they work together to catalyze the respective modifications on downstream target RNAs (Wang et al., 2016).

Demethylases

Demethylases perform a reverse process to that described above and demethylate the mRNA modified with m6A and hence also known as an eraser. Thus far, demethylases included two reported proteins: fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5)(Jia et al., 2011; Zheng et al., 2013). FTO, belonging to the AlkB family of non-heme Fe (II)/dioxygenases, was the first identified demethylase of m6A in RNAs (Jia et al., 2011). FTO contributes to the regulation of mRNA alternative splicing by modulating m6A levels (Batista et al., 2014; Zhao et al., 2014). ALKBH5, also belonging to the AlkB family, has been identified as a demethylase for m6A modification of RNAs (Zheng et al., 2013). ALKBH5 regulates mRNA export, RNA metabolism, and assembly of mRNA processing factors in nuclear speckles (Zheng et al., 2013).

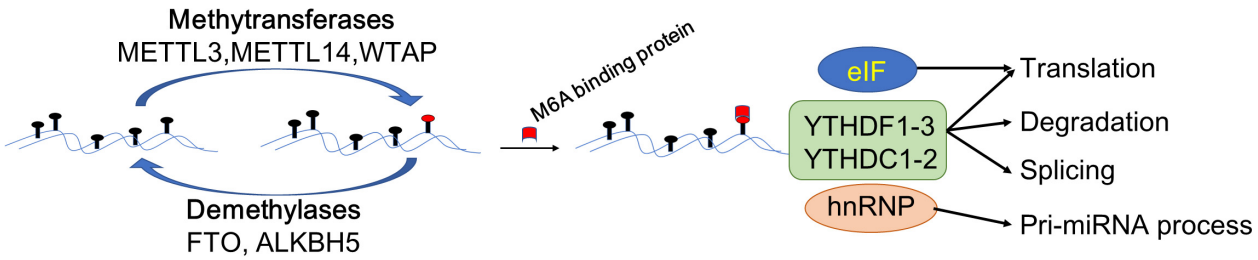

FIGURE 1 | Schematic elucidation of m6A modification in the regulation of gene expression. The m6A modification is catalyzed by methyltransferases METTL3/14, Wilms tumor 1-associating protein (WTAP). The demethylases fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (FTO/ALKBH5) demethylated the bases modified by m6A. Methylated reader proteins recognize the mRNA modified by m6A, thereby activating downstream pathways by different reader proteins. After modified by methyltransferases, eIF3 proteins promote mRNA translation. HNRNPA2B1 regulates the processing of the pre-miRNA and pri-miRNA. Further, YTHDF1-3, YTHDC1-2 can regulate the processes of RNA translation, degradation, and splicing.

Methylated Reader Proteins

The reversible chemical modification requires the recognition of the m6A-modified RNAs by reader proteins such as YTH domain proteins, nuclear heterogeneous ribonucleoprotein (hnRNP), and eukaryotic initiation factors (eIF), which are involved in the translation, degradation, and miRNA processing of downstream targets in the pathway (Wang X. et al., 2014). The fate of m6A-RNA is varied and even "contradictory." For example, in different target RNAs, m6A modification can promote both the translation and degradation processes of mRNA, which is determined by the m6A reading protein "reader" (**Figure 1**). Previous studies showed that recognition by YTHDF1/3 promoted the translation process of mRNA (Shi et al., 2017; Han et al., 2020), whereas recognition by YTHDF2 induced mRNA degradation process (Zhao et al., 2017; Lai et al., 2018). Diversely, eIF3 proteins are mainly bound to the 5′ untranslational region (5′-UTR) of RNAs to promote mRNA translation (Skabkin et al., 2015), while hnRNPA2/B1, which is one of the hnRNP proteins, recognizes target m6A-RNAs, activates the downstream pathway of the pri-miRNA, and regulates the processing of the pre-miRNA (Alarcon et al., 2015).

Functions of the m6A Process

M6A Participates in Physiological Activities

The m6A modification influences the downstream pathways by regulating the fate of an RNA transcript, processing, splicing, degradation, or translation, whether mRNA or non-coding RNA(Fazi and Fatica, 2019; Zhang et al., 2020). It plays an important role in various biological processes at different levels of the m6A modification of RNA, such as circadian clock (Fustin et al., 2013, 2018; Zhong et al., 2018), DNA damage response (Xiang et al., 2017; Zlotorynski, 2017), neural function regulation (Poeck et al., 2016; Zhang F. et al., 2017), drosophila sex determination (Haussmann et al., 2016; Poeck et al., 2016), and embryonic development (Kwon et al., 2019).

For example, PER2 and Bmal1 were discovered as the clock genes that control the pace of our daily lives to maintain the human circadian clock (Lowrey and Takahashi, 2011). Casein Kinase 1 Delta mRNA (Ck1δ) encodes a critical kinase that controls circadian rhythms by enhanced translation of PER2, which is negatively regulated by m6A (Zhong et al., 2018). When m6A is inhibited, CK1δs levels are increased, and the increased stabilization of the PER2 protein, as a result, leads to a slower clock (Zhong et al., 2018). Zhong et al. (2018) also found that the m6A modification was involved in the regulation of the circadian clock through the clock gene Bmal1 (Fustin et al., 2018). Bmal1 affects the levels of m6A modification and controls the expression of PPARα to regulate lipid metabolism. These findings revealed a new way by which the circadian clock regulates metabolism. Another study found that m6A modulates sex determination in drosophila (Haussmann et al., 2016). As Sxl (Sex-lethal) is a switch gene involved in sex determination, the m6A modification of the pre-mRNA of Sxl, affected its selective splicing and thereby the regulation of drosophila sex development (Haussmann et al., 2016).

M6A Participates in the Pathological Processes of Diseases

The m6A modification also causes diseases such as neurodevelopmental delay (Li H.B. et al., 2017; Yoon et al., 2017), immunodeficiency (Li H.B. et al., 2017), and male infertility (Zheng et al., 2013; Yang et al., 2016). Based on current evidence, the findings and investigations on m6A function provide a new direction for the treatment of these diseases. The m6A modulates murine spermatogenesis; after the inactivation of m6A methyltransferases, the level of m6A modification significantly reduced, which could lead to sperm formation disorder (Li H.B. et al., 2017). m6A methylation is also involved in regulating testosterone synthesis in Leydig cells (LCs); the study on m6A methylation provides a new direction for the treatment of azoospermia and oligospermia (Chen et al., 2020). Li H.B. et al. (2017) found that m6A modification controlled T cell homeostasis by targeting the IL-7/STAT5/SOCS pathways. After the knockout of METTL3, m6A modification in T cells decreased, thus impairing their ability to differentiate. Consequently, these T cells could not cause autoimmune diseases, providing a new way to alleviate autoimmune diseases with drugs that target m6A modification (Li H.B. et al., 2017).

M6A Participates in the Development of Malignant Tumors

It was not surprising, therefore, to find that m6A modification was involved in the occurrence and development of different types of malignant tumors (Deng et al., 2018; Lai et al., 2018; Wei et al., 2019). m6A modification affects tumor proliferation, differentiation, tumorigenesis, invasion, and metastasis by regulating proto-oncogenes and tumor suppressor genes. The translation of the m6A modified gene was changed, which affected the development and progression of the tumor. For example, in lung cancer, METTL3 promotes cell growth and leads to cancer by increasing the expression of EGFR and TAZ (Lin et al., 2016). In human hepatocellular carcinoma, knockdown of METTL3 decreased SOCS2 mRNA modification and increased SOCS2 mRNA expression, suppressing the progression of liver cancer (Chen et al., 2018). Recently, it has been demonstrated that m6A methylation participates in the self-renewal and pluripotent regulation of stem cells, even in CSCs (Zhang S. et al., 2017; Wu et al., 2018). To explore the underlying role of m6A in the differentiation of CSCs, the next part of this review focuses on research related to m6A function in CSCs.

ROLE OF m6A IN THE DIFFERENTIATION OF CSCs

CSCs in Leukemia

The Role of Leukemia Stem Cells (LSCs) in the Occurrence of Myeloid Leukemia

Typically, HSCs differentiate into myeloid progenitors and eventually mature myeloid cells (Nishikii et al., 2017). Dysregulation of this process results in the development of diseases such as acute myeloid leukemia (AML), an aggressive clonal disease of abnormal HSCs, and primitive progenitors that blocks their myeloid differentiation to generate self-renewing leukemia stem cells (LSCs) (Testa, 2011). Furthermore, the presence of leukemia stem/initiating cells (LSCs/LICs) can lead to the occurrence or relapse of myeloid leukemia, which is likely to be a major cause of drug-resistant disease and relapse in AML patients (ten Cate et al., 2010).

M6A in the Processes of Hematopoiesis

Recently, it has been revealed that m6A participates in the process of endothelial hematopoietic transition (EHT), which is the mechanism underlying HSCs generation (Thambyrajah et al., 2016). In invertebrates, the Notch signaling pathway is critical to the development of hematopoietic stem and progenitor cells (HSPCs) during embryogenesis (Robert-Moreno et al., 2008). Zhang et al. demonstrated that G protein-coupled receptor 183 (Gpr183) signaling repressed Notch signaling before the onset of EHT, serving as an indispensable switch for HSPC emergence, and the inhibition of Gpr183 abolished HSPC emergence by significantly upregulating Notch signaling (Zhang et al., 2015). Another investigation revealed that in zebrafish, the stability of notch 1 was mediated by METTL3 through m6A modification and recognized by YTHDF2 to maintain the balance of gene expression during the EHT process, thus regulating the fate of HSCs (Zhang C. et al., 2017). Generally, METTL14 can be suppressed by SPI1, which plays an essential role in generating early myeloid progenitors (Weng et al., 2018). As expected, the critical role of METTL3 and METTL14 in normal and malignant hematopoiesis was proved, and there is evidence that the expression levels of METTL3 and METTL14 are highly increased in HSPCs and decreased during normal differentiation (Vu et al., 2017; Weng et al., 2018).

To explore the physiological functions of YTHDF2, Li et al. used the conditional mouse model of *Ythdf2* knockout and found that the number of functional HSCs increased without skewing lineage differentiation or causing hematopoietic malignancies. This demonstrates the physiological functions of YTHDF2 in adult stem cell maintenance by regulating the stabilities of mRNAs critical for self-renewal of HSCs (Li et al., 2018).

M6A in the Processes of Leukemogenesis

The proto-oncogenes MYB and MYC are reported to be overexpressed in many human malignant tumors including AML and contribute to disease progression by inhibiting differentiation and promoting self-renewal of AML cells (Gonda and Metcalf, 1984; Bahr et al., 2018). Ramsay et al. reported the aberrant expression of METTL14 in AML cells and its involvement in the regulation of the expression of MYB and MYC through m6A-based post-transcriptional regulation, indicating the critical role of METTL14 in the self-renewal of LSCs/LICs and development of AML (Ramsay and Gonda, 2008). Vu et al. (2017) demonstrated the oncogenic role of m6A in myeloid leukemia by promoting the translation of c-MYC, BCL2, and PTEN mRNAs (**Figure 2**).

For treating hematological disorders including cancer, transplantation of HSCs from human umbilical cord blood (hUCB) holds great application foreground but has restrictive uses because of limited numbers (Gincberg et al., 2018). In hematopoietic malignancies, the expression of YTHDF2 in leukemia patients was significantly increased, suggesting the potential promoting function of YTHDF2 in the occurrence and development of leukemia (Paris et al., 2019). In animal models, it was found that suppressing YTHDF2 expression can significantly inhibit the leukemia process and prolong the survival period of tumor-bearing mice, indicating that YTHDF2 is very important to the development of leukemia (Paris et al., 2019). Paris et al. (2019) pointed out that YTHDF2 was not essential for normal HSC function; however, increased expression of YTHDF2 was required for both initiation and propagation of AML, contributing to the integrity of LSC function by decreasing stabilities of m6A transcripts including the tumor necrosis factor receptor *Tnfrsf2*. Importantly, the upregulation of *Tnfrsf2* in *Ythdf2*-deficient LSCs primed malignant cells for apoptosis, predicting YTHDF2 as a potential therapeutic target in patients with AML to selectively inhibit LSCs and promote the expansion of HSCs (Paris et al., 2019).

CSCs in Solid Tumors

In addition to the role of m6A in the regulation of the differentiation of normal hematopoietic process and leukemia hematopoietic process, recent evidence focuses on the role of

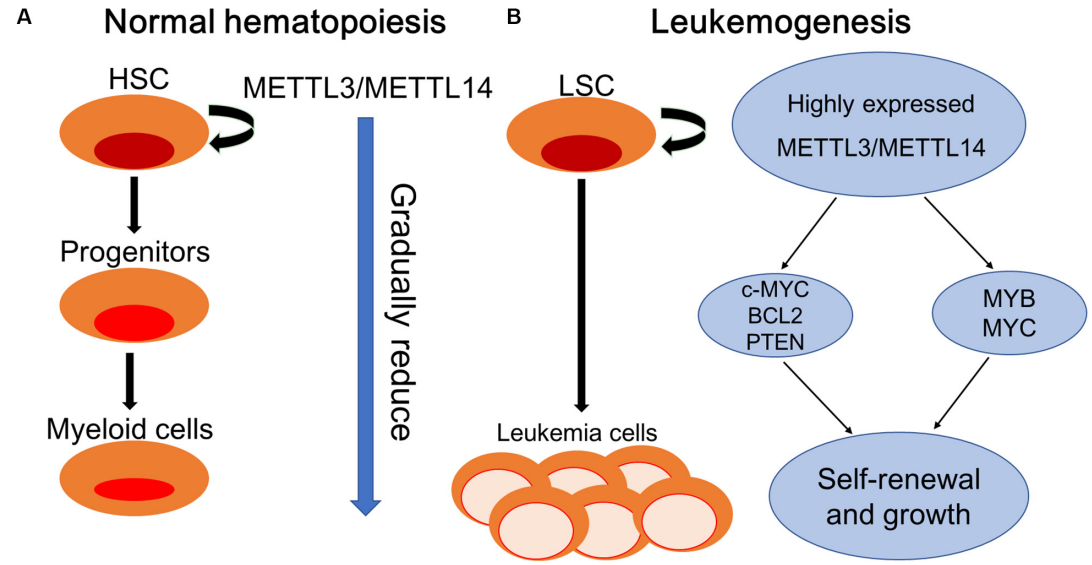

FIGURE 2 | Schematic elucidation of normal hematopoiesis and leukemogenesis. **(A)** Normal hematopoiesis: hematopoietic stem cells (HSCs) differentiate into myeloid progenitors and eventually mature myeloid cells. METTL3/METTL14 are highly expressed in hematopoietic stem and progenitor cells (HSPCs) and are downregulated during normal differentiation. **(B)** Leukemogenesis: myeloid differentiation of HSPCs is blocked to produce self-renewing LSCs. METTL3/METTL14 are aberrantly expressed in leukemia stem cells (LSCs). METTL3 promotes the self-renewal and growth of LSCs by enhancing the translation of c-MYC, BCL2, and PTEN mRNAs. METTL14 promotes self-renewal and growth through the induction of MYB and MYC mRNA translation.

TABLE 1 | The reported roles of M6A enzymes in CSCs of solid tumors.

CSCs	M6A enzyme	Targets	Reported Function	References
BCSC	ALKBH5	NANOG	Increase the percentage of BCSCs	Zhang C. et al., 2016; Zhang C. Z. et al., 2016
GSCs	METTL3	SOX2	Enhance radiation resistance	Visvanathan et al., 2018
	ALKBH5	FOXM1	Enhance self-renewal and tumorigenesis	Zhang S. et al., 2017
	FTO	ADAM19	Enhance GSC growth and self-renewal	Cui et al., 2017
CCSCs	YTHDF1	Wnt/β-catenin pathway	Enhance colonosphere self-renewal and suppresses differentiation	Bai et al., 2019

CSCs, cancer stem cells; BCSCs, breast cancer cells; GSCs, glioblastoma stem cells; CCSCs, colorectal cancer stem cells.

m6A in regulating tumorigenesis in solid tumors by affecting the fate of CSCs (**Table 1**).

Breast Cancers

Breast CSCs (BCSCs), with their infinite proliferative ability through self-renewal and transient amplifying cells, play important roles in tumor growth, motility, invasion, metastasis, and resistance to chemotherapy (Beretov et al., 2018). Oskarsson et al. (2014) systematically reviewed the sources, niches, and vital pathways of metastatic stem cells and elucidated that metastasis in malignant tumors was powered and initiated by disseminated cancer cells with survival, self-renewal, dormancy, and reactivation abilities, namely, metastatic stem cells (MetSCs). Interestingly, the existence of BCSCs was originally described as of hematopoietic origin (Al-Hajj et al., 2003; Mani et al., 2008; Pece et al., 2010), and the MetSCs were capable of reinitiating distant tumor growth, independent of the origin or phenotypic characteristics of primary tumors (Oskarsson et al., 2014). Certain cytokines were proven to stimulate CSC features and that BCSC potential was promoted by transforming growth factor β (TGF-β) in synergy with the Wnt signaling pathway (Scheel et al., 2011). Zhang et al. (2009, 2013) found that abnormal CXCL12/IGF1 signaling and Src activities in patients with breast tumors predicted an increased risk of bone relapse. It is accepted that the phenotype of BCSCs is distinct and specified by the expression of core pluripotency factors including Kruppel-like factor 4 (KLF4), OCT4, SOX2, and NANOG (Hu et al., 2008; Yu et al., 2011; Leis et al., 2012; Iv Santaliz-Ruiz et al., 2014), providing potential effective therapeutic strategies for patients with breast cancer to eliminate BCSCs (Oskarsson et al., 2014).

It is accepted that breast cancer involves intratumoral regions under hypoxic conditions with activated hypoxia-inducible factors (HIFs) during the development process, and in response to hypoxia or chemotherapy, HIFs induce the BCSCs phenotype accordingly, which is implicated in resistance to chemotherapy, disease recurrence, and metastasis (Xiang and Semenza, 2019). Using animal models (Zhang C. et al., 2016) found that HIF-induced expression of ALKBH5, an m6A demethylase, promoted the BCSCs phenotype by demethylating and increasing the mRNA levels of NANOG, a pluripotency factor. Soon after, the same group demonstrated another molecular mechanism

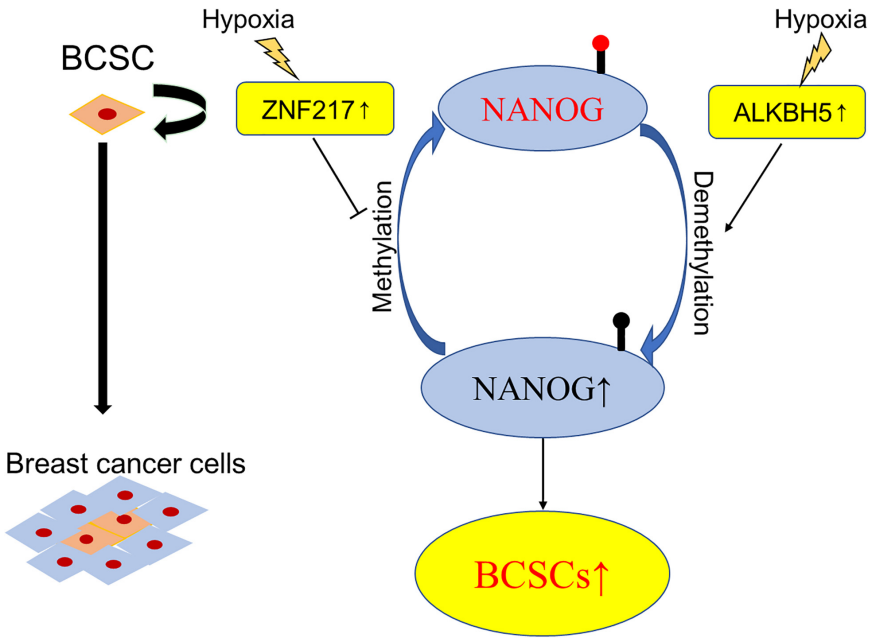

FIGURE 3 | Schematic elucidation of m6A in BCSCs. Exposure of breast cancer cells to hypoxia can induce ALKBH5-mediated demethylation of NANOG mRNA, leading to increased expression of NANOG, and increasing the percentage of BCSCs.

of HIF-induced pluripotency with BCSCs specification, namely, zinc finger protein 217 (ZNF217)-dependent inhibition of m6A methylation of NANOG and KLF4 (Zhang C. Z. et al., 2016). These findings verified the participation of m6A modification in the differentiation of BCSCs and provided novel therapeutic targets for breast cancer patients, especially in the hypoxic tumor microenvironment (**Figure 3**).

Glioblastoma (GBM)

Glioblastoma (GBM) is the most prevalent and lethal primary tumor in the brain, with invasion into the surrounding brain structures. Conventional therapeutic strategies include surgery, radiotherapy, and adjuvant chemotherapy (Stupp et al., 2009). Although targeted therapies or immunotherapies are reported to fight GBM to improve the survival and quality of life of GBM patients, an efficient cure was never achieved (Diaz et al., 2017). Lathia et al. (2015) systematically reviewed the role of CSCs in GBM and demonstrated that self-renewing and tumorigenic abilities of CSCs contributed to tumor initiation and therapeutic resistance.

Cui et al. (2017) verified the critical role of m6A modification in the self-renewal and tumorigenesis of glioblastoma stem cells (GSCs) by artificially modifying the expression of METTL3 or METTL14, the key components of the RNA methyltransferase complex, *in vitro* and *in vivo*. Knockdown of METTL3 or METTL14 enhances GSCs growth and self-renewal, in contrast, an FTO inhibitor suppresses the progression of GSC-initiated tumors (Cui et al., 2017). The abnormal expression of ALKBH5 was also detected in GSCs, to demethylate FOXM1 nascent transcript and enhance the expression of Forkhead box protein M1 (FOXM1) (Zhang S. et al., 2017). Interestingly, a long non-coding RNA FOXM1-AS (antisense to FOXM1) promotes the interaction between ALKBH5 and FOXM1 transcript and GSC tumorigenesis through the FOXM1 axis (Zhang S. et al., 2017). As the FOXM1 and adamalysin-19 (ADAM19) have proved to play oncogenic roles in malignant tumors (Nandi et al., 2018; Wang et al., 2019a), the above-mentioned evidence proved the oncogenic function of the m6A demethylases ALKBH5 and FTO in enhancing self-renewal and tumorigenesis through the regulation of FOXM1 and ADAM19, respectively (Cui et al., 2017; Zhang S. et al., 2017).

However, the diverse roles of m6A modification in GSC have been reported recently. Visvanathan et al. found a high level of entire METTL3-mediated m6A modification, associated with the maintenance of stem-like cells and the dedifferentiation of glioma cells (Visvanathan et al., 2018). Further experiments revealed that the pluripotency factor SOX2 was the m6A target of METTL3, and it was stabilized by recruiting Human antigen R (HuR) to m6A-modified SOX2 mRNA, resulting in decreased sensitivity to γ-irradiation (Visvanathan et al., 2018). In addition to the evidence that SOX2 was associated with radiation resistance in various cancers (Lee et al., 2015), the recruitment of HuR binding to m6A-modified transcripts was found to be preferential and global (Visvanathan et al., 2018), suggesting that other target genes of m6A modification may be involved in the regulation of irradiation sensitivity. These findings suggested that mRNA m6A levels seem opposite, predicting the diverse targets and functions of m6A modification in different processes of malignant tumors, such as tumorigenesis and radiation resistance, and suggesting the potential target role of m6A modification for the treatment of GSCs (**Figure 4**).

Colorectal Cancer (CRC)

Colorectal cancer (CRC), the second most common cause of cancer-related death in the United States, is generally treated with combined application of surgery, radiation, and chemotherapy (Siegel et al., 2020). However, with the recurrence of metastasis, it results in treatment failure, which is currently a major challenge. Colorectal CSCs (CCSCs) are reported to be the main causes of recurrence and metastasis in CRC patients (Wang et al., 2020). Since (Dalerba et al., 2007) identified CSCs in CRC in 2007, great efforts have been made to explore the underlying mechanism of the regulation of these cells in CRC and revealed special molecular pathways involved in CCSCs regulation, such as the Wnt/β-catenin pathway (Ordonez-Moran et al., 2015) and Notch signaling (Jin et al., 2017).

The function of m6A modification in CCSCs has also raised concern among researchers. To explore the role of YTHDF1 in CRC, Bai et al. overexpressed YTHDF1 in CRC and found that YTHDF1 can promote the tumorigenicity and xenograft tumor growth of cells in CRC *in vitro* and *in vivo*, respectively (Bai et al., 2019). Further investigation verified that overexpression of the reader protein YTHDF1 promoted colonosphere formation and self-renewal, thought inhibiting Wnt/β-catenin pathway activities in cells in CRC, while knockdown the expression of YTHDF1, inhibited colonosphere self-renewal while enhancing their differentiation (Bai et al., 2019). Although research is limited, the regulatory function of YTHDF1 in CCSCs evokes further investigations on the regulation of CSCs activities and their therapeutic targets for CRC patients.

Osteosarcoma

Osteosarcoma is a malignant bone tumor that has a high prevalence in adolescents and children, with a high mortality rate (Schneiderman et al., 1984). Although osteosarcoma is potentially initiated from a single cell as a monoclonal disease, the quick development of a polyclonal disease position it as one of the most complex cancers in terms of molecular aberration (Brown et al., 2017). Gibbs et al. (2005) first identified and reported osteosarcoma stem cells (OSCs) based on the expression of Oct 3/4, Nanog, and STAT3 in bone sarcoma cells, serving as potential targets for selective noncytotoxic therapy in bone sarcoma patients, which are rather resistant to current therapeutic protocols. OSCs play a central role in chemoresistance and in metastasis, which is the main cause of cancer-related death in patients with osteosarcoma (Yan et al., 2016).

Recently, the m6A modification and gene expression differences in OSCs were detected through m6A MeRIP-seq and RNA-seq and, and it was found that m6A-related enzymes, METTL3, METTLE14, and ALKBH5, were abnormally expressed in OSCs (Wang et al., 2019b). Importantly, the differentially methylated genes were enriched in signaling pathways regulating the pluripotency of stem cells and correlated with the poor prognosis in patients with osteosarcoma (Wang et al., 2019b). The m6A modification may be a breakthrough mechanism to improve the treatment of osteosarcoma and provide a fundamental contribution to the search for new therapeutic targets for OS (Wang et al., 2019b).

IMPLICATIONS FOR CANCER THERAPIES

The Treatment Strategies Targeting CSCs

As CSCs in malignant tumors provide a new therapeutic strategy for cancer treatment, four main CSC-targeted therapies directed at stem cell fate regulation are currently under development and investigation (Ahmad and Amiji, 2017; Pan et al., 2018).

(1) Antibodies targeting surface markers of CSCs. Based on the identification of specific surface markers for CSCs, such as $CD34^{+}/CD38^{-}$, CD33, and $CD44^{+}/CD24^{-}$ (Al-Hajj et al., 2003; ten Cate et al., 2010), antibodies against specific surface markers have been developed and even used in clinical settings. For example, as 80–90% of stem cells in AML express CD33, antibodies targeting CD33, such as gemtuzumab, became an important drug for the treatment of AML (Laing et al., 2017).

(2) Target drugs to CSC-related pathways. Series of abnormal activation of signaling pathways in CSCs, such as PI3K/Akt/mTOR, Wnt, and Notch, and other signaling pathways have been detected in different types of malignant tumors (Bertacchini et al., 2015; Takebe et al., 2015; Venkatesh et al., 2018); providing targeted therapy to these signaling pathways has also become an important therapeutic strategy. For example, the antitumor drugs rapamycin and everolimus, targeting the PI3K/Akt/mTOR signaling pathway, have been evaluated in the treatment of leukemia; however, further clinical studies are required (Bertacchini et al., 2015).

(3) Inducing the differentiation of CSCs. Compared with normal cells, the differentiation of CSCs is either abnormal or blocked (Pan et al., 2018). Therefore, inducing the differentiation and maturation of CSCs provides a useful and potential method to block their ability to self-renewal and effectively inhibit tumor growth (Han et al., 2015). Presently, 90% of the patients with acute promyelocytic leukemia were completely relieved by all-trans vitamin A acid-induced differentiation (Ohno et al., 2003).

(4) Changing the microenvironment of CSCs (Prasetyanti and Medema, 2017). It was revealed that the abnormal microenvironment transforms normal stem cells into CSCs, leading to the formation of malignant tumors (Liu and Fan, 2015). Therefore, restoring the tumor microenvironment to the normal one is particularly important to provide the potential of reversing CSC differentiation.

Targeting CSC Therapies Associated With m6A Enzymes

Rhein is the first identified natural inhibitor of FTO (Chen et al., 2012). Niu et al. (2019) found that Rhein can inhibit breast cancer cell proliferation, colony formation, and metastasis *in vitro* and *in vivo*. However, the activity and specificity of these FTO inhibitors are relatively poor, and their mechanism of action has not been fully studied. MA2, the ethyl ester form of meclofenamic acid (MA), was recently identified as a selective inhibitor of FTO (Huang et al., 2015). MA2 was used in the treatment of GSCs and was effective in the *in vitro* and *in vivo* experiments, while

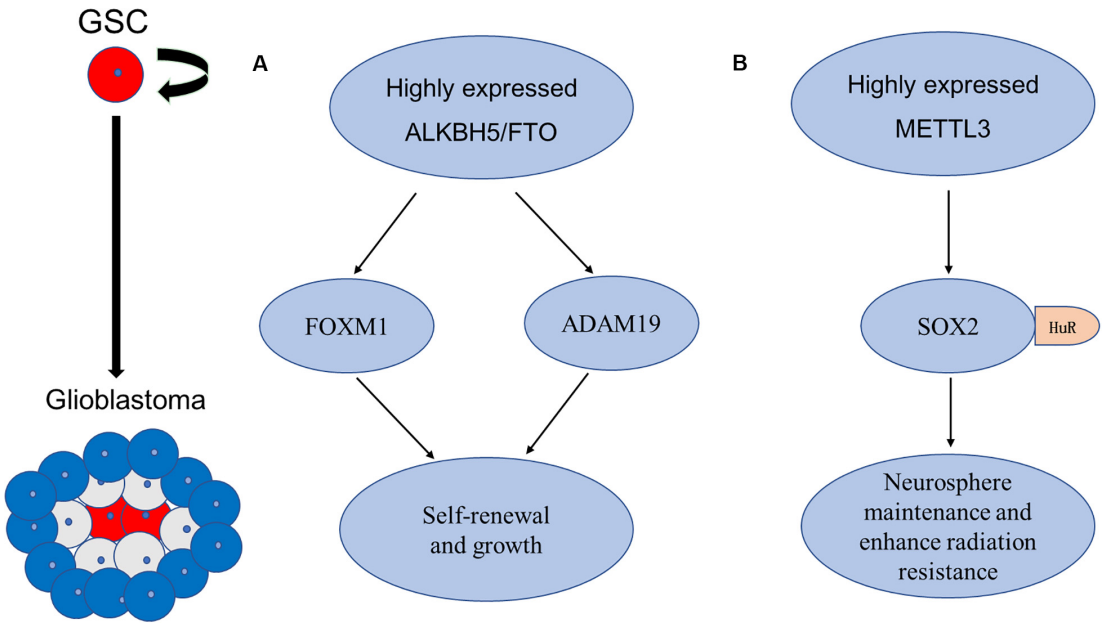

FIGURE 4 | Schematic elucidation of m6A in GSCs. **(A)** Cui et al. and Zhang et al. found that RNA m6A demethylase ALKBH5 and FTO are aberrantly expressed in GSCs, and they enhance self-renewal and tumorigenesis of GSCs through regulation of FOXM1 and ADAM19, respectively. **(B)** Visvanathan et al. found that METTL3 is upregulated in GSCs. METTL3-mediated methylation in SOX2 mRNA through recruitment of HuR to enhance its stability in GSCs, the increased level of the SOX2 enhanced radiation resistance.

the RNA m6A demethylases ALKBH5 and FTO enhanced self-renewal and tumorigenesis of GSCs (Cui et al., 2017). In GSC-grafted animals, MA2 suppressed glioblastoma progression and prolonged the lifespan of GSC-grafted animals (Cui et al., 2017).

Therefore, more effective FTO inhibitors need to be developed for clinical application. Recently, Huang et al. reported two new small molecule inhibitors of FTO, namely, FB23 and FB23-2, directly binding to FTO and specifically inhibiting the activity of m6A demethylase of FTO, finally resulting in the suppression of AML cell proliferation (Huang et al., 2019). Because of the reported oncogenic role of FTO in AML (Li Z. et al., 2017), their inhibitors such as FB23 and FB23-2 are expected to have a potential treatment effect in AML patients, and future potential for use in the clinic. However, drugs targeting m6A modification of CSCs in malignant tumors are limited, and further investigations are needed to explore potential targets and drugs in this field.

SUMMARY AND PERSPECTIVES

Cancer stem cells, with self-renewal and tumorigenesis abilities, are the major cause for tumor recurrence and chemotherapy resistance. However, the underlying mechanisms have not been fully elucidated. Recent studies revealed the regulating role of m6A in the differentiation of CSCs. The present review focused on this field to review the function and regulating role of m6A modification in the differentiation of CSCs, especially to explore the potential mechanism underlying the determination of their fates. Currently, inhibitors of FTO and ALKBH5 can be used as candidates for anticancer drug development; especially to inhibit the growth of cancer cells by manipulating their m6A modification levels. Although these inhibitors have not been tested in clinical trials yet, they provide more possibilities for early diagnosis and treatment of cancer.

AUTHOR CONTRIBUTIONS

H-TW contributed conception and design of the study and revised the original manuscript critically. YX, JL, and H-TW organized the database, searched the literature, and structured and drafted the manuscript, figures, and table carefully. W-JC, Q-QY, W-TC, and C-LL organized the database and partially drafted the manuscript carefully. All authors contributed to manuscript revision, and read and approved the submitted version.

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REVIEW
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Recent Advances in Liver Cancer Stem Cells: Non-coding RNAs, Oncogenes and Oncoproteins

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Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, with high morbidity, relapse, metastasis and mortality rates. Although liver surgical resection, transplantation, chemotherapy, radiotherapy and some molecular targeted therapeutics may prolong the survival of HCC patients to a certain degree, the curative effect is still poor, primarily because of tumor recurrence and the drug resistance of HCC cells. Liver cancer stem cells (LCSCs), also known as liver tumor-initiating cells, represent one small subset of cancer cells that are responsible for disease recurrence, drug resistance and death. Therefore, understanding the regulatory mechanism of LCSCs in HCC is of vital importance. Thus, new studies that present gene regulation strategies to control LCSC differentiation and replication are under development. In this review, we provide an update on the latest advances in experimental studies on non-coding RNAs (ncRNAs), oncogenes and oncoproteins. All the articles addressed the crosstalk between different ncRNAs, oncogenes and oncoproteins, as well as their upstream and downstream products targeting LCSCs. In this review, we summarize three pathways, the Wnt/β-catenin signaling pathway, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, and interleukin 6/Janus kinase 2/signal transducer and activator of transcription 3 (IL6/JAK2/STAT3) signaling pathway, and their targeting gene, c-Myc. Furthermore, we conclude that octamer 4 (OCT4) and Nanog are two important functional genes that play a pivotal role in LCSC regulation and HCC prognosis.

Keywords: hepatocellular carcinoma, liver cancer stem cells, non-coding RNAs, oncogenes, oncoproteins

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INTRODUCTION

Among tumor types, liver cancer is the third leading cause of death in humans around the globe (Torre et al., 2015; Forner et al., 2018). Hepatocellular carcinoma (HCC) is one of the most common subclass accounting for 90% of liver cancer (Bruix et al., 2014). Most HCC patients are no longer eligible for curative treatment, such as transplantation or surgical resection, because of disease progression to the late stage. Simultaneously, while molecular targeted therapies and chemotherapy are available for partial HCC patients, clinical benefits remain unsatisfactory. As a result, exploration of new systemic treatment approaches for HCC is important due to poor outcomes (Blum, 2005; Forner et al., 2012). Interestingly, using surface markers, studies have identified cancer stem cells (CSCs) and isolated CSC subpopulations from HCC cells in the field of liver CSCs (LCSCs) (Liu Y.M. et al., 2015). Although LCSCs only represent a small subset of liver cancer cells, they are considered to be responsible for HCC tumorigenesis, progression, metastasis

and recurrence. Therefore, many scholars have conducted studies on LCSCs. In addition, scholars have summarized their results from the perspective of genes or RNA. However, new research results are constantly emerging. To provide LCSC researchers with more information, this review will summarize studies newly reported from the perspective of ncRNAs, oncogenes and oncoproteins.

THE ROLE AND CLASSIFICATION OF ncRNAs

The main function of RNA is to bridge the transformation process from genetic information to translation of genetic information into proteins. In transcriptional precursor RNA, more than 70% of the genome is transcribed into non-coding RNAs (ncRNAs), only approximately 20% of which are transcribed into messenger RNA (mRNAs). The common feature of ncRNAs is that they can be transcribed from the genome but perform their respective biological functions at the RNA level without being translated into proteins. There is sufficient evidence to demonstrate the important role of ncRNAs in regulating LCSCs. ncRNAs include microRNAs (miRNAs), long ncRNAs (lncRNAs), small interfering RNAs (siRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and small nuclear RNAs (snRNAs). Here, we will focus on the most studied members of the ncRNA family, miRNAs and lncRNAs.

miRNAs Associated With LCSCs

MicroRNA is a subset of the ncRNA family that can regulate expression of more than 60% of human genes. While miRNA is a group of short ncRNAs containing approximately 22 nucleotides and are not completely complementary to target mRNAs, they inhibit post-transcriptional translation by binding to the 3′-untranslated region (3′-UTR) of target mRNAs (DeSano and Xu, 2009; Gargalionis and Basdra, 2013). Moreover, dysregulation of miRNA expression is linked to tumorigenesis in humans (Budhu et al., 2008; Ji et al., 2009a; Li et al., 2010; Fu et al., 2014) and regulates the stemness features of CSCs (Ji et al., 2009b). The characteristics of several miRNAs whose expression is associated with LCSCs are well known, such as miR-130b (Ma et al., 2010), miR-21 (Tomimaru et al., 2010; Zhou et al., 2013), miR-214 (Xia et al., 2012), miR-425-3p (Vaira et al., 2015), and miR-517a (Toffanin et al., 2011). Here, we summarize the recently identified miRNAs whose deregulation enhances or suppresses LCSC properties (**Supplementary Figure 1**).

miRNAs That Enhance LCSC Properties

miR-429

E2F transcription factor 1 (E2F1) has been found to be a novel regulator of pluripotent stem cells (Yeo et al., 2011). Interestingly, the protein-protein interaction between E2F1 and RB transcriptional co-repressor 1 (RB1) was significantly weakened upon transfection with miR-429. Moreover, miR-429 can modulate the transcriptional activity of E2F1 via direct targeting of RB binding protein 4 (RBBP4). Furthermore, the stemness-related gene OCT4 was identified as an E2F1-responsive gene and was upregulated upon RBBP4 silencing or high miR-429 expression. In sum, high expression of miR-429 contributed to self-renewal, tumorigenicity, proliferation and chemoresistance in HCC. In addition, miR-429 was found to target a novel functional axis, RBBP4/E2F1/OCT4, to manipulate HCC (Li L. et al., 2015).

miR-1246

Glycogen synthase kinase 3β (GSK3β) and axis inhibition protein 2 (AXIN2) are negative regulators of Wnt signaling and are tumor suppressors in HCC (Reya and Clevers, 2005). A recent study demonstrated that miR-1246 promotes tumorigenesis, metastasis and chemoresistance of LCSCs by activating the Wnt/β-catenin signaling pathway. Mechanistically, an *in silico* prediction indicated that AXIN2 and GSK3β were potential downstream targets of miR-1246. Interestingly, miR-1246 activated the Wnt/β-catenin pathway by suppressing GSK3β and AXIN2 expression, which are key members of the β-catenin destruction complex. Furthermore, OCT4 was the direct upstream regulator of miR-1246, which activated miR-1246 expression through miR-1246 promoter binding and cooperatively drove β-catenin activation in LCSCs (Chai et al., 2016).

miR24-2

miR24-2 can promote tumorigenesis by epigenetically enhancing the tyrosine kinase Src and can epigenetically regulate liver cancer by altering the expression of various Histone H3/4 epigenetic modifications in LCSCs. Moreover, histone deacetylase 3 (HDAC3), Nanog and PI3K were found to be key players in the signaling pathways mediated by miR24-2. Furthermore, miR24-2 targeted the protein arginine methyltransferase 7 (PRMT7) 3′-UTR and inhibited PRMT7 expression, thereby reducing the bi/trimethylation of histone H4R3. Importantly, miR24-2 promoted the transcriptional activity and maturation of the miR675 precursor (pri-miR675) through binding to Nanog in LCSCs. lncRNA HULC plays a key role in the carcinogenesis triggered by miR24-2. Moreover, miR24-2-dependent PI3K activation promoted autophagy (Wang L. et al., 2019).

miR-199a-3p, miR-155

Transforming growth factor beta 1 (TGF-β1) has been confirmed to be an important enhancer of CSCs and epithelial-mesenchymal transition (EMT) (Polyak and Weinberg, 2009). miR-199a-3p plays an important role and is upregulated in LCSCs. Consistently, overexpression of TGF-β1 and hepatitis B virus X (HBx) have been associated with LCSC properties and poor prognosis in hepatitis B virus (HBV)-related liver cancer. TGF-β1 cooperation with HBx can activate the c-Jun N-terminal kinase (JNK)/c-Jun pathway, while miR-199a-3p, a regulator of hepatic progenitor cell (HPC) transformation, can be activated by c-Jun. In conclusion, TGF-β1/HBx co-regulated the miR-199a-3p signaling axis targeting malignant transformation of HPCs (Dong et al., 2019). Furthermore, miR-155 overexpression promoted cell EMT in liver cancer cells, and overexpression of miR-155 promoted the stemness of LCSCs via down-regulation of tumor protein P53 inducible nuclear protein 1 (TP53INP1), which is a downstream target gene of miR-155. In addition, in vitro, TGF-β1 indirectly downregulated TP53INP1 expression via miR-155 upregulation in liver cancer cells (Ji et al., 2015; Liu Y.M. et al., 2015; Liu et al., 2015a,b).

miR-500a-3p and miR-589-5p

Evidence has indicated that the JAK/STAT signaling pathway acts as a critical regulator in several well-known CSCs (Jove, 2000). One study reported that miR-500a-3p promotes CSC properties by targeting suppressor of cytokine signaling (SOCS)2, SOCS4 and protein tyrosine phosphatase non-receptor type 11 (PTPN11) through STAT3 signaling activation (Jiang et al., 2017). Another study found that overexpression of miR-589-5p decreased overall and relapse-free survival in HCC. Further mechanistic analysis revealed that miR-589-5p activated the STAT3 pathway by inhibiting its negative regulators. Moreover, upregulation of miR-589-5p enhanced LCSC properties (Long et al., 2018).

Furthermore, zinc finger e-box binding homeobox (ZEB)1/2 is a key transcription factor in EMT, and is the most prominent target of the miR-200 family (Burk et al., 2008). Deregulation of miR-200b was involved in regulation of LCSCs, the miR-200b–ZEB1 circuit was found to regulate diverse LCSCs (Tsai et al., 2017), and miR-219 down-regulated E-cadherin via its mRNA 3′UTR, thus playing a role in the sensitivity of HCCs to sorafenib (Si et al., 2019), miR-137 expression was upregulated in CD44-positive CSCs and found to be associated with a significantly shorter survival periods for HCC patients (Sakabe et al., 2017).

miRNAs That Suppress LCSC Properties

miR-125b

Increasing evidence suggests that EMT contributes to metastasis and recurrence in HCC (Choi and Diehl, 2009). Zhou et al. (2015) found that overexpression of miR-125b could attenuate migration, chemoresistance and LCSC generation by suppressing EMT. Moreover, they revealed that miR-125b suppressed EMT by targeting small mothers against decapentaplegic (SMAD)2 and SMAD4. These findings suggest that ectopic expression of miR-125b is a potential HCC treatment strategy (Zhou et al., 2015).

miR-192-5p

miR-192-5p was found to be significantly down-regulated in LCSCs. Suppression of miR-192-5p markedly increased LCSC numbers and the features of LCSCs through targeting of poly(A) binding protein cytoplasmic 4 (PABPC4). The axis of tumor protein p53 (TP53) mutation/mir-192 promoter hypermethylation/reduced miR-192-5p/increased PABPC4 was identified in HCCs expressing high levels of CSC markers. These findings reveal a genetic regulatory signaling pathway shared by different LCSCs (Gu et al., 2019).

miR-302a/d

miR-302a/d negatively regulates spheroid formation and cell growth and promotes apoptosis of liver cancer cells by suppressing the targeted E2F transcription factor 7 (E2F7) gene. In one study, miR-302a/d inhibited LCSC cell cycle entry and self-renewal via targeting the E2F7/Akt axis. These results suggest that miR-302a/d and E2F7 might be potential biomarkers of LCSCs (Ma et al., 2018).

miR-26b-5p

Epithelial cell adhesion molecule (EpCAM) is one of the most prevalent LCSC markers. Recently, researchers reported that miR-26b-5p targets both heat shock protein family A member 8 (HSPA8) and EpCAM. Reduced expression of miR-26b-5p enhanced LCSC invasion, migration and tumorigenesis. Moreover, miR-26b-5p was responsible for maintaining EpCAM-positive LCSCs by targeting of HSPA8 (Khosla et al., 2019).

Furthermore, miR-1305 overexpression reversed the suppressor that inhibited LCSC properties by suppressing the ubiquitin-conjugating enzyme E2T (UBE2T)-dependent Akt-signaling pathway (Wei et al., 2019). While knockdown of miR-25 enhanced the sensitivity of LCSCs to TNF-related apoptosis inducing ligand (TRAIL)-mediated apoptosis via the phosphatase and tensin homologue (PTEN)/PI3K/Akt/Bad signaling pathway (Feng et al., 2016). miR-365 directly regulated Ras-related C3 botulinum toxin substrate 1 (RAC1) by binding with the mRNA 3′UTR and affected HCC drug resistance (Jiang et al., 2019). In addition, miR-486 directly targeted sirtuin1, which exhibits high expression in self-renewing and tumorigenic LCSCs (Yan et al., 2019).

LncRNAs Associated With LCSCs

Long ncRNAs are a subclass of ncRNAs longer than 200 nucleotides. They have emerged as critical epigenetic regulators of gene expression and share some characteristics of mRNAs (Devaux et al., 2015). lncRNAs exert their functions via diverse mechanisms, including cytoplasmic complexes, modulation of gene expression, nuclear scaffolding, transcriptional regulation and pairing with other RNAs (Ulitsky and Bartel, 2013). lncRNAs can regulate gene expression through chromosome remodeling, transcription and post-transcriptional processing. Dysregulation of lncRNA expression has been associated with widespread development of many cancers (Zhang M. et al., 2016; Xiaoguang et al., 2017). We summarize the latest deregulated lncRNAs that enhance or suppress LCSC properties (**Supplementary Figure 2**).

LncRNAs That Enhance LCSC Properties

lncTCF7 and lnc-β-Catm

lncTCF7 can regulate transcription factor 7 (TCF7) expression by recruiting the SWI/SNF complex in the nuclei of LCSCs. Then, the TCF7 expression triggers Wnt signaling to initiate self-renewal of LCSCs. In sum, lncTCF7-mediated Wnt signaling primes LCSC self-renewal and tumor propagation (Wang et al., 2015). In addition, a study revealed a new transcribed lncRNA called lncRNA β-catenin methylation (lnc-β-Catm), which could also regulate self-renewal of LCSCs. Moreover, lnc-β-Catm was responsible for inhibiting β-catenin ubiquitination, allowing β-catenin to activate Wnt–β-catenin signaling and sustaining the stemness of LCSCs (Zhu et al., 2016a).

DANCR

In one study, genome-wide analyses identified tumor-associated lncRNA-DANCR. Dysregulation of DANCR was explored in HCC tumorigenesis and colonization. The activation of DANCR was confirmed to be associated with poor survival of HCC patients. Recently, Yuan et al. (2016) reported that lncRNA-DANCR was overexpressed in LCSCs. Experiments showed that knockdown of DANCR decreased stem-cell properties and tumor cell vitality. In further mechanistic studies, DANCR associated

with Catenin Beta 1 blocked the repressive effect of miR-2214, miR-199a, and miR-320a (Yuan et al., 2016).

lncBRM

LINCR-0003 (lncBRM) was overexpressed in LCSCs and maintained their self-renewal stemness properties via Yes-associated protein 1 (YAP1) signaling. In addition, lncBRM associated with Brahma (BRM) initiated the BRM/SWI2-related gene 1 (BRG1)/BRM switch. Next, the BRG1-associated factor complex activated YAP1 signaling. Furthermore, lncBRM expression with the addition of YAP1 signaling was associated with the prognosis of HCC (Zhu et al., 2016b).

HAND2-AS1

INOsitol-requiring 80 (INO80) chromatin-remodeling complex, which is a conserved complex that modifies chromatin using the energy of adenosine triphosphate (ATP), controls gene expression and maintains stem cell properties (Ayala et al., 2018). One study revealed that lncRNA HAND2-AS1 expression was upregulated in LCSCs. Importantly, HAND2-AS1 recruited the INO80 complex to bone morphogenetic protein receptor type 1A (BMPR1A), inducing bone morphogenetic protein (BMP) signaling activation. Mechanistically, overexpression of lncRNA HAND2-AS1 associated with the INO80 complex can promote the self-renewal of LCSCs and drive liver oncogenesis (Wang et al., 2019b).

CUDR

Cancer upregulated drug resistant (CUDR) is a new ncRNA gene that is highly expressed in HCC. A study revealed that decreased phosphatase and tensin homolog (PTEN) might enhance the binding ability of CUDR to Cyclin D1. In this study, the CUDR-Cyclin D1 complex loaded onto the lncRNA H19 promoter region enhanced H19 expression. Moreover, the CUDR-Cyclin D1-CTC-binding factor (CTCF) complex promoted c-Myc expression (Pu et al., 2015). SET1A is a component of the histone methyltransferase complex. One study found that SET-domain-containing 1A (SET1A) cooperated with CUDR to promote malignant transformation of hepatocyte-like SCs (Li et al., 2016). Furthermore, research has shown that CUDR is highly upregulated in liver cancer and can cause abnormal β-catenin signaling during malignant transformation of LCSCs (Gui et al., 2015).

lncHOXA10

HOXA10 (homeobox A10) is a member of the HOX transcription factor family, which is highly expressed in liver tumors. HOXA10 interacts with some signaling pathways and participates in many types of cancer (Cui et al., 2014; Li et al., 2014). Recently, a study found that HOXA10 is upregulated during liver tumorigenesis and tumor-initiating cell (TIC) self-renewal. The authors found that both lncHOXA10 and HOXA10 were highly expressed and participated in self-renewal regulation in liver cancer and liver TICs. lncHOXA10 interacts with NURF chromatin remodeling complex and binds to the HOXA10 promoter to drive transcription initiation (Shao et al., 2018).

Furthermore, lncRNA HCG11 regulates insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) to inhibit apoptosis of HCCs via mitogen-activated protein kinase (MAPK) signaling transduction (Xu et al., 2017), and lncZic2 drives the self-renewal of liver TICs via the myristoylated alanine rich protein kinase C substrate (MARCKS) and MARCKS like 1 (MARCKSL1) (Chen et al., 2018). Moreover, lncRNA n339260 (Zhao et al., 2018) and lncCAMTA1 (Ding et al., 2016) were suggested to be new prognostic biomarkers of LCSCs.

LncRNAs That Suppress LCSC Properties

lnc-DILC

The suppressor lnc-DILC resides in both the nucleus and cytoplasm. A recent study showed the subcellular distribution of lnc-DILC and revealed its nuclear localization in LCSCs. Likewise, it was determined that lnc-DILC could depress IL-6 transcription and regulate LCSC expansion by suppressing IL-6 autocrine signaling. Interestingly, knockdown of lnc-DILC affected IL-6 transcription, STAT3 activation and LCSC expansion. Nuclear factor kappa B (NF-κB) was found to be an essential link between inflammation and cancer (Ben-Neriah and Karin, 2011) and to play a pivotal role in CSC maintenance (Kagoya et al., 2014). In another recent study, the authors clarified a paradigm of LCSC expansion in which lnc-DILC functions as a novel link connecting tumor necrosis factor (TNF)-a/NF-κB signaling with the autocrine IL-6/STAT3 cascade (Wang X. et al., 2016).

DLX6-AS1

lncRNA distal-less homeobox 6 antisense 1 (DLX6-AS1) belongs to the DLX gene family (Wang P. et al., 2017). One study demonstrated that DLX6-AS1 is highly expressed in HCC and serves as an oncogene targeting the DLX6-AS1/miR-203a/matrix metallopeptidase 2 (MMP-2) pathway (Zhang et al., 2017). Intriguingly, DLX6-AS1 can promote the stemness of osteosarcoma cells by regulating miR-129-5p/delta like non-canonical notch ligand 1 (DLK1) (Zhang et al., 2018). Additionally, cell adhesion molecule 1 (CADM1) expression was downregulated and facilitated tumorigenesis in HCC (Zhang W. et al., 2016). Another recent study showed that suppression of DLX6-AS1 inhibited tumorigenesis through the STAT3 signaling pathway, which restrains CADM1 promoter methylation in LCSCs (Wu et al., 2019).

THE ROLE OF ONCOGENES OR ONCOPROTEINS ASSOCIATED WITH LCSCS

Current evidence indicates that during hepatocarcinogenesis, one potential pathogenic mechanism is abnormalities in oncogenes or oncoproteins. Interestingly, oncogenes play an important role in cell growth, proliferation and division (Hinds et al., 1989; Rochlitz et al., 1993). Genes with deletions, insertions or mutations may lose their functions and are related to cancer development. A large number of experiments have shown that abnormalities in oncogenes or the expression of oncoproteins are implicated in oncogenesis, tumor progression and metastasis

by targeting LCSCs. According to GeneCards[1], which shows the localization of human genes, gene subcellular locations will be described based on compartments as follows: nucleus, cytoplasm, and plasma membrane, among others (**Supplementary Table 1**).

Oncogenes or Oncoproteins Mainly Located in the Nucleus of Human Cell

Sox9

The sex determining region Y box 9 (Sox9) protein is predominantly localized in the nucleus of HCCs. Sox9 is a transcription factor that is expressed in several cancers (Guo et al., 2012; Sarkar and Hochedlinger, 2013). Sox9 is significantly highly expressed in HCC and associated with decreased survival. Consistently, the proportion of Sox9 knockdown cells in S and G2/M phases was reduced and that in G0/G1 phase was increased. Furthermore, the expression of Sox9 was coincident with expression of the LCSC markers CD13 and OCT4. Knockdown of Sox9 expression in LCSCs cells resulted in a reduction in the expression of the stem cell transcription factors B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1), OCT4 and Nanog, as well as in α-fetoprotein and β-catenin. Additionally, Sox9 was decreased during asymmetrical cell division and regulated the asymmetrical-to-symmetrical cell division switch in LCSCs (Liu C. et al., 2016).

MacroH2A1

Macrohistone H2A (MacroH2A) is a subclass of the H2A family containing two isoforms, encoded by macroH2A1 and macroH2A2. The MacroH2A1 gene is associated with tumorigenesis in many cancer types (Gaspar-Maia et al., 2013; Borghesan et al., 2016). Interestingly, macroH2A1 can protect differentiated HCC cells from chemotherapeutics as a marker (Rappa et al., 2013; Borghesan et al., 2016). A recent study found that downregulation of macroH2A1 enhanced the expression of stemness-related genes and hypoxia factor. Furthermore, depletion of macroH2A1 activated the phosphorylated nuclear factor kappa B p65 pathway, which is responsible for inducing LCSCs (Lo Re et al., 2018b). Knockdown of macroH2A1 led to LCSC-like features and massive alterations to the nuclear architecture in HCCs (Douet et al., 2017). MacroH2A1-depleted cells showed two changes in lipid metabolism and glucose in LCSCs: massive acetyl-coA upregulation, which transformed lipid content; and increased activation of the pentose phosphate pathway, which provides precursors for nucleotide synthesis. macroH2A1 was also found to rewire lipid and carbohydrate metabolism in HCC toward LCSCs (Lo Re et al., 2018a).

REX1

REX1 is also called zinc finger protein 42 (ZFP42) (Jiang et al., 2002) and has been studied in multiple cancer types (Kim et al., 2011). Steve TLUK et al. found that REX1 was frequently downregulated in HCC tumors. Furthermore, they explored the possibility that REX1 silencing was regulated by promoter hypermethylation, histone methylation and histone acetylation in human HCC. In addition, silencing of REX1 potentiated the tumorigenesis and metastasis potential of HCC. The molecular mechanism by which REX1 deficiency enhanced the stemness appeared to involve p38 MAPK signaling regulation in a mitogen-activated protein kinase kinase 6 (MKK6)-dependent manner (Luk et al., 2019). Furthermore, REX1 silencing promoted F-actin reorganization and changed oxidative stress levels through a p38 MAPK-dependent pathway.

MYCN

MYCN is a member of the MYC family, which comprises basic helix–loop–helix–zipper transcription factors. MYCN is one of the central regulators of the growth-promoting signal transduction that maintains stem-like properties (Takahashi and Yamanaka, 2006). Acyclic retinoid (ACR) is capable of preventing HCC recurrence in hepatitis C virus (HCV)-positive patients who have undergone curative removal of primary tumors (Muto et al., 1996). Recent research found that ACR significantly inhibited MYCN expression at both the gene and protein level. Mechanistically, MYCN is expressed at high levels in S and G2 phases in cells. Knockdown of MYCN repressed cell cycle progression and induced cell death. Furthermore, MYCN expression was correlated with EpCAM, Alpha-fetoprotein (AFP), and CD133 expression and activated Wnt/β-catenin signaling in HCC (Qin et al., 2018).

ZFX

Zinc finger protein X-linked (ZFX) is a zinc finger transcription factor encoded on the mammalian X chromosome and is frequently upregulated in various malignancies (Jiang and Liu, 2015; Li Y. et al., 2015). One study demonstrated that high ZFX expression conferred self-renewal and chemoresistance properties to HCC cells by binding of the SRY-box transcription factor (Sox)2 and Nanog (Lai et al., 2014). Recently, Chao Wang et al., reported that ZFX expression in LCSCs was relevant to poor prognosis. Consistently, silencing ZFX expression suppressed tumorigenicity and the metastatic potential of $EpCAM^+$ LCSCs *in vitro*. Interestingly, knockdown of ZFX suppressed the expression of several β-catenin target genes, such as cyclin D1, c-Jun and c-Myc. More importantly, ZFX was responsible for maintaining stem-like features of $EpCAM^+$ LCSCs by facilitating β-catenin nuclear translocation and transactivation (Wang C. et al., 2017).

HOXB7

Homeobox B7 (HOXB7) belongs to the homeobox gene family, which plays a role in some solid tumors (Chile et al., 2013; Joo et al., 2016). EMT causes epithelial cells to lose their cell-cell adhesions, plays an important role in HCC metastasis (Candini et al., 2015). A previous study showed that HOXB7 enhanced the proliferation and self-renewal of LCSCs (Care et al., 1999). A recent investigation showed that HOXB7 was highly expressed in HCC cells and could facilitate growth and metastasis of cell stemness and EMT, correlating with poor prognosis. Further mechanistic research suggested that HOXB7 promoted metastasis by activating the Akt pathway to upregulate c-Myc and Slug in HCC. In conclusion, HOXB7 promotes EMT

[1]https://www.genecards.org/

and modulates the PI3K/Akt/c-Myc axis to facilitate stem cell pluripotency in HCC (Huan et al., 2017).

Tcf7l1

The β-catenin-transcription factor 7 like 1 (Tcf7l1) shows high expression in many malignant tumors and has a crucial effect on the Wnt/β-catenin pathway (Murphy et al., 2016). However, another study reported the opposite results, finding that Tcf7l1 expression was down-regulated in LCSCs and associated with poor survival of HCC patients. Further mechanistic research showed that Tcf7l1 attenuation upregulated the expression of stemness genes, including kruppel like factor (KLF)4, OCT4 and Nanog, and down-regulated the expression of differentiation genes, including glucose-6-phosphatase (G6p), albumin and transthyretin. Tcf7l1 knockdown further impacted the protein expression of Nanog. Moreover, Tcf7l1 phosphorylation and protein degradation through the mitogen-activated protein kinase (MEK)/extracellular signal regulated kinase (ERK) pathway were negatively regulated by extracellular insulin-like growth factor (IGF) signaling (Shan et al., 2019).

Furthermore, Sox12 is a potential marker in LCSCs (Zou et al., 2017). Another a transcription factor, E26 transformation-specific transcription factor ELK3 (ELK3), is activated by mitogen-activated protein kinase-associated signaling pathways (Buchwalter et al., 2005). The expression of ELK3 was upregulated in $CD133^+/CD44^+$ HCC cells. Furthermore, silencing the expression of ELK3 in $CD133^+/CD44^+$ LCSCs could downregulate their metastatic potential by modulating hypoxia inducible factor 1α (HIF-1α) expression (Lee J.H. et al., 2017). In addition, forkhead box M1 (FOXM1) belongs to the forkhead box protein family, which plays an important role in DNA replication, mitosis and genomic stability (Laoukili et al., 2005). FOXM1 inhibited LCSC proliferation, migration, invasion, colony formation and EMT by promoting apoptosis. Furthermore, silencing of FOXM1 suppressed the expression of Sox2, OCT4, and Nanog in LCSCs by decreasing the expression of acetaldehyde dehydrogenase-2 (Chen et al., 2019). In addition, ring finger protein 1 (Ring1), an essential cofactor of polycomb group proteins, was upregulated in HCC and targeted p53 to promote cancer cell proliferation (Xiong et al., 2015; Shen et al., 2018). Zhu et al. (2019) found that overexpression of Ring1 activated the Wnt/β-catenin signaling pathway and drove malignant transformation of LCSCs. In addition, KLF8, which belongs to the KLF family of transcription factors (Pearson et al., 2008), is highly expressed in LCSCs, and KLF8 gene silencing suppressed the invasion and migration of LCSCs. For the further mechanism, Wnt/β-catenin signaling participates in the KLF8 regulation process (Shen et al., 2017).

Oncogenes or Oncoproteins Mainly Located in Both the Nucleus and Cytoplasm of Human Cells

Shp2

Src-homology 2 domain–containing phosphatase 2 (Shp2) is a non-receptor protein tyrosine phosphatase encoded by PTPN11 (Feng et al., 1993). Studies have demonstrated that Shp2 highly expression is associated with poor prognosis in various malignancies (Aceto et al., 2012; Han et al., 2015). A recent study found that upregulation of Shp2 facilitated expansion by promoting self-renewal of LCSCs. Further research on the mechanism revealed that Shp2 dephosphorylated cell division control protein 73 in the cytosol of hepatoma cells and that Shp2 could augment nuclear accumulation of β-catenin. Furthermore, Shp2 increased β-catenin accumulation by inhibiting the glycogen synthase kinase GSK3β in LCSCs (Xiang et al., 2017).

ZIC2

Zic family member 2 (ZIC2) belongs to the zinc finger transcription factor gene family (Benedyk et al., 1994). A previous study showed that ZIC2 was enhanced in various tumors and regulated tumorigenesis (Marchini et al., 2012). Bromodomain PHD finger transcription factor (BPTF) is the largest subset of the nuclear remodeling factor (NURF) chromatin remodeling complex (Li et al., 2006). The NURF complex is responsible for embryonic differentiation, development and stemness maintenance (Cherry and Matunis, 2010). A recent study demonstrated that ZIC2 expression was high in LCSCs and could regulate their self-renewal. Mechanistically, ZIC2 can bind to the upstream region of OCT4 and initiate its activation. Importantly, ZIC2 interacts with the NURF complex in the nucleus of HCCs. Furthermore, ZIC2 silencing abolished its binding capacity to the NURF complex, but depletion of the NURF complex did not affect the binding capacity of ZIC2 to the OCT4 promoter. These findings suggest that the NURF complex regulates OCT4 expression directly. In sum, ZIC2 can sustain the stemness of LCSCs by recruiting the NURF complex to trigger OCT4 activation (Zhu P. et al., 2015).

BPTF

The NURF complex can also modulate chromatin structure by targeting genes that make transcription factors more accessible (Song et al., 2009). One study reported that BPTF could activate oncogenic signaling and synergize with other proteins to regulate tumor progression (Dar et al., 2016; Richart et al., 2016). Another recent study reported high BPTF expression in HCC. In addition, down-regulation of BPTF expression affected cell colony formation, proliferation, chemotherapy resistance and apoptosis and tumor progression in HCC. However, human telomerase reverse transcriptase (hTERT), a catalytic subset of the telomerase holoenzyme complex, synthesizes telomeres using its own RNA as a template and then adds the telomeres to the ends of chromosomes (Liu N. et al., 2016). Further study of the molecular mechanisms showed that BPTF promotes tumor cell proliferation, tumor metastasis and stemness maintenance by activating hTERT expression in HCCs (Zhao et al., 2019).

IRAK1

Interleukin-1 receptor-associated kinase 1 (IRAK1) phosphorylation is implicated in tumorigenesis (Dussiau et al., 2015). However, the role of IRAK1 itself in TICs and HCC is not clear. In a recent study, Cheng et al. (2018) found that overexpression of IRAK1 in HCC was related to poor

prognosis. Importantly, IRAK1 was found to regulate self-renewal, tumorigenicity, chemoresistance and TIC expression in HCC. Mechanistically, knockdown of IRAK1 revealed that Aldo-Keto Reductase Family 1 Member 10 (AKR1B10) was a target of IRAK1 mediated through activator protein 1 (AP-1) activation. More importantly, IRAK1 augmented stemness and chemoresistance through AP-1/AKR1B10 signaling in HCC (Cheng et al., 2018).

BORIS

BORIS is the paralog of CCCTC-binding factor (CTCF), also called CCCTC-binding factor-like (CTCFL) (Marshall et al., 2014). Notably, increasing evidence shows that BORIS is expressed in CSCs and associated with CSC-like properties (Alberti et al., 2014, 2015). In one study, Liu et al. (2017) found that BORIS overexpression increased CD90 expression, drug resistance, migration, invasion and stem cell marker (Sox2, OCT4, and c-Myc) expression in human HCC cells. Mechanistically, BORIS regulates OCT4 via epigenetic modification, with changes in the histone methylation status of the OCT4 promoter at CTCF sites. BORIS maintains an active chromatin conformation via increasing the histone 3 lysine 4 bimethylation (H3K4me2)/histone H3 lysine 27 trimethylation (H3K27me3) ratio to enhance OCT4 expression (Liu et al., 2017).

TARBP2

Transactivation response element RNA-binding protein 2 (TARBP2) is a double-stranded RNA-binding protein governing the translation of mRNA (Gatignol et al., 1991). TARBP2 was suggested to be a potential regulatory factor in CSCs (De Vito et al., 2012). The study identified that restoration of TARBP2 expression resensitized HCC to sorafenib. TARBP2-mediated sensitization of HCC to sorafenib was miRNA-independent. Interestingly, TARBP2 protein was destabilized by autophagic-lysosomal proteolytic degradation in HCC cells. Mechanistically, downregulated TARBP2 expression promoted sorafenib resistance via stabilization of Nanog expression and increased LCSC properties in HCC cells (Lai et al., 2019).

Oncogenes or Oncoproteins Mainly Located in the Cytoplasm of Human Cells

iNOS

An increasing number of studies suggest that NO, which is produced by inducible NO synthase (iNOS), promotes tumor initiation (Granados-Principal et al., 2015; Davila-Gonzalez et al., 2017). Additionally, the Notch signaling pathway can promote CSC self-renewal, migration, differentiation, proliferation and survival in several malignancies (Androutsellis-Theotokis et al., 2006). A recent study reported that iNOS exhibited high expression in $CD24^{+}/CD133^{+}$ LCSCs. Furthermore, iNOS/NO was associated with aggressive human HCC by activating the Notch signaling pathway. The Notch signaling activation was dependent on upregulation of iRhom-2 and 3′,5′-cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-mediated activation of transarterial chemoembolization (TACE). These studies provide a mechanism explaining the tumorigenic effects of iNOS in LCSCs and indicate that targeting iNOS could have therapeutic benefits in HCC (Wang et al., 2018).

GLS1

Glutaminase 1 (GLS1), which converts glutamine to glutamate, is associated with proliferation, growth and metabolism in cancer cells (Aledo et al., 2000). Previous studies have demonstrated that GLS1 is responsible for cell invasion and migration, which predict a poor prognosis in HCC (Yu et al., 2015). GLS1 mRNA has been reported to generate two isoforms, with the shorter form named glucose absorption capacity (GAC) and the longer form called α-ketoglutaric acid (KGA) (Elgadi et al., 1999). In a recent report, Yitao Ding et al., reported that both the KGA and GAC isoforms were exclusively located in the mitochondrial matrix. In addition, the mitochondrial matrix protein GLS1 is highly expressed in LCSCs. Mechanistically, targeting GLS1 or glutamine metabolism increased reactive oxygen species (ROS) accumulation, which suppressed β-catenin translocation from the cytoplasm to the nucleus, leading to a decrease in stemness-related gene expression. GLS1 regulates the stemness features of LCSCs via ROS/Wnt/β-catenin signaling (Li B. et al., 2019).

KIF15

Kinesin family member 15 (KIF15) plays an important role in many malignant tumors with a tetrameric spindle motor structure (Reinemann et al., 2017; Sheng et al., 2019). Nevertheless, the mechanism by which KIF15 targets LCSCs remains unclear. A recent study found that KIF15 was highly expressed in HCC tissues from patients with higher recurrence and shorter overall survival. Experimentally, low ROS levels in the tumor microenvironment have been verified to support the stemness of CSCs (Lee K.M. et al., 2017). KIF15 can promote LCSC stemness. Further mechanistic research showed that KIF15 markedly decreased intracellular ROS levels and increased the LCSC phenotype via phosphoglycerate dehydrogenase (PHGDH). Furthermore, the chromatin-associated protein ANCCA (also known as ATAD2, the ATPase family AAA domain-containing protein 2) appears to have an important role in enhancing KIF15 expression (Li Q. et al., 2019).

ANXA3

Annexin A3 (ANXA3), which belongs to the annexin family of $Ca2^{+}$-dependent phospholipid-binding proteins, has the ability to promote tumorigenesis and resistance to chemotherapy (Raynal and Pollard, 1994; Pan et al., 2015). Stephanie Ma et al., found that high expression of both secretory and endogenous ANXA3 was correlated with HCC pathogenesis. They further found that secretory ANXA3 could be detected in sera of HCC patients and that the secretory ANXA3 played a crucial role in maintenance of LCSC-like properties. Mechanistically, exogenous ANXA3 was internalized via caveolin-1-dependent endocytosis. In addition, exogenous ANXA3 overexpression resulted in c-Jun N-terminal kinase (JNK) pathway activation, as evidenced by increased c-Myc expression, reduced p21 expression and increased JNK activity. In sum, ANXA3 is responsible for enhancing stemness in $CD133^{+}$ LCSCs via the JNK pathway (Tong et al., 2015).

Cygb

Cytoglobin (Cygb) is a member of the human hexacoordinate hemoglobin family. Cygb is a tumor suppressor whose deficiency contributes to tumor recurrence and poor prognosis in multiple malignancies (Xu et al., 2013; Thuy le et al., 2016). Oxidative-nitrosative stress (ONS) is an independent etiologic factor in HCC tumorigenesis (Wang Z. et al., 2016). Accumulating evidence indicates that the interaction of ONS with CSCs promotes tumorigenesis, progression, and hemoradiotherapy resistance (Su et al., 2016). A recent study found that Cygb was deregulated in HCC tissue and the decrease aggravated the growth of LCSCs. Furthermore, Cygb absence promoted LCSC phenotypes and PI3K/Akt activation in HCC progression but inhibited HCC proliferation and LCSC stemness in an ONS-dependent manner (Zhang et al., 2019).

Oncogenes or Oncoproteins Mainly Located in the Cell Membrane in Human Cells

NUMB

NUMB is a tumor suppressor and cell fate determinant, and loss of NUMB expression has been observed in cancer (Colaluca et al., 2008). The p53-NUMB complex was independently demonstrated to be a tumor suppressor (March et al., 2011). Recently, study showed that NUMB phosphorylation plays a crucial role in tumor-initiating cell self-renewal and liver tumorigenesis via the Nanog pathway. Further mechanistic research, Nanog increased phosphorylation of NUMB and decreased p53 by modulating the atypical protein kinase C zeta/Aurora A kinase (aPKCf-AURKA) pathway, which is an upstream pathway for NUMB phosphorylation. Furthermore, the phosphorylation of NUMB by Nanog destabilized the NUMB-p53 complex, leading to destabilization of p53 and subsequent high self-renewal of TICs (Siddique et al., 2015).

AQP3

Aquaporin 3 (AQP3) is a member of the water channel protein family, which can be found in the plasma membranes of various cells (Verkman, 2012). Studies have shown that aberrant AQP3 expression contributes to several malignant tumors (Huang X. et al., 2017; Xiong et al., 2017). Accumulating evidence supports the notion that AQP3 is related to maintain of CSC stemness (Zhou et al., 2016). Recently, Yawei Wang and his colleagues reported that AQP3 expression was high in HCCs. Additionally, depletion of AQP3 suppressed the proliferation and invasion of $CD133^{+}$ HCC. In addition, AQP3 promoted LCSC properties by regulating STAT3 nuclear translocation and phosphorylation (Wang et al., 2019a).

ITGA7

Integrins are a subclass of glycoproteins that mediate cell-cell or cell-extracellular adhesion (LaFlamme et al., 2018). Integrin alpha 7 (ITGA7) was demonstrated to maintain stemness through targeting CSC biomarkers in various cancers (Ming et al., 2016). Recently, Ge et al. (2019) found that knockdown of ITGA7 suppressed proliferation, reduced CSC marker expression levels (CD44, CD133, and OCT4) and enhanced apoptosis by targeting the protein tyrosine kinase 2 (PTK2)-PI3K-Akt signaling pathway in liver cancer cells. However, overexpression of ITGA7 promoted proliferation and suppressed apoptosis but not CSC marker expression via the PTK2-PI3K-Akt signaling pathway. Then, they further performed compensation experiments, which verified that ITGA7 regulates cell stemness through the PTK2-PI3K-Akt signaling pathway (Ge et al., 2019).

CD44s, CLDN1, and FZD2

Some oncogenes are located in cell junctions, the cell membrane, and the basolateral cell membrane and have a common mechanism for targeting EMT. Increasing evidence suggests that EMT is connected with CSC properties and cancer metastasis and recurrence (Choi and Diehl, 2009). A previous study reported that the isoform switch to CD44s was essential for cells to undergo EMT (Brown et al., 2011). Recently, Asai et al. (2019) investigated the roles of CD44s in LCSCs. Knockdown of CD44s expression resulted in decreased spheroid formation and increased drug sensitivity. In addition, another study reported that CD44s is involved in maintenance of LCSCs via the notch receptor 3 (NOTCH3) signaling pathway (Asai et al., 2019). Moreover, claudin 1 (CLDN1) plays a critical role in the EMT process in HCC (Suh et al., 2017). However, transmembrane protease serine 4 (TMPRSS4) is a contributing mediator during EMT and an inducer of the CSC phenotype in multiple tumors (Huang et al., 2014; de Aberasturi et al., 2016). Mahati et al. (2017) observed that TMPRSS4 and CLDN1 were remarkably upregulated in HCC tissues, while overexpression of CLDN1 induced EMT and CSC behaviors via TMPRSS4 in HCC. Mechanistically, Ou et al. (2019) provided evidence that Frizzled 2 (FZD2) is a driver of EMT and CSC properties in HCC.

Oncogenes or Oncoproteins in Other Locations or Pathways in Human Cells

RACK1, Tg737, and MAGE-A9

Some oncogenes are located in many parts of the cell, for example, the cell membrane, cytoplasm, cytoskeleton, perinuclear region, nucleus, cell projections, dendrites, and phagocytic cups. In the same manner, they can target different targets and ultimately affect the stemness of LCSCs. Receptor for activated C kinase 1 (RACK1) belongs to the Trp-Asp repeat protein family and is an adaptor protein involved in multiple signaling pathways (Bourd-Boittin et al., 2008). Overexpression of RACK1 is associated with short overall survival and a high recurrence rate in HCC (Ruan et al., 2012). In recent work, RACK1 was found to directly stabilize Nanog, thus contributing to the self-renewal and chemoresistance of LCSCs (Cao et al., 2019). In addition, the Tg737 gene is a mouse intra-flagellar transport 88 homologue that was first identified in Chlamydomonas (Pazour et al., 2000). Previous studies have shown that Tg737 expression highly suppresses LCSC properties. Consistently, Tg737 gene silencing was significantly associated with tumor differentiation, metastasis, and invasion and alpha-fetoprotein levels (You et al., 2017). Furthermore, knockdown of Tg737 caused liver cancer cells to acquire LCSC properties during malignant transformation, because Tg737 regulated a double-negative feedback loop between Wnt/β-catenin and hepatocyte

nuclear factor 4-alpha, resulting in EMT (Huang Q. et al., 2017). Moreover, the melanoma antigen gene (MAGE) family represents one of the largest groups of human tumor-associated antigens. MAGE-A9, a member of the MAGE-A gene family, is frequently expressed in various human tumors (Gu et al., 2014). MAGE-A9 contributes to malignant biological phenotypes, including cell proliferation, chemoresistance and migration of $EpCAM^+$ HCC cells (Wei et al., 2018).

OPN, CCN3, and LOX

A subset of secretory oncogenes can localize in many parts of the cell. Osteopontin (OPN) is a subclass of phosphorylated glycoproteins and is associated with chemoresistance in many malignant tumors (Pang et al., 2011; Hsieh et al., 2013). Considerable evidence has revealed that OPN enhances the CSC phenotype in cancer (Pietras et al., 2014). Guoke Liu et al., reported that secreted OPN induced autophagy by sustaining forkhead box O3a (FoxO3a) stability and binding with its integrin. The autophagy promoted LCSC properties and chemoresistance (Liu G. et al., 2016). Another study found that down-regulation of OPN expression in $CD133^+/CD44^+$ cells suppressed migration and proliferation by regulating DNA methyltransferase (DNMT)1 expression. Downregulation of DNMT1 expression reduced global DNA methylation. Additionally, various levels of OPN exhibited different sensitivities to 5 Aza (Gao et al., 2018). Moreover, cellular communication network factor 3 (CCN3) is associated with the malignant phenotype of HCC. Furthermore, one study found that CCN3 overexpression enhanced survival and increased in vivo metastasis of HCC. Mechanically, CCN3 affects the upregulation of OPN and coagulation factors, which led to enhance stemness of LCSCs (Jia et al., 2017). Lysyl oxidase (LOX) is a secreted enzyme, that contributes to regulation of various factors, including extracellular matrix (ECM) maintenance, migration and angiogenesis (Zhu J. et al., 2015; Ribeiro et al., 2017). A recent study revealed that LOX gene expression was upregulated in cell spheres and led to more vascular enrichment in a mouse xenograft model. Furthermore, LOX expression increased vascular endothelial growth factor (VEGF) and enhanced the tube formation capacity of endothelial cells. These findings provide a novel mechanism of LOX in regulation of TICs in HCC (Yang et al., 2019).

CONCLUSION

Hepatocellular carcinoma is a solid cancer with high morbidity and mortality. Evidence has shown that the existence of LCSCs can contribute to HCC tumor initiation, drug resistance, metastasis and recurrence. Intriguingly, LCSC elimination seems to be an ideal method to defeat HCC. Therefore, specific targeting of LCSCs may repress the malignant biological behaviors of HCC and improve curative effects. Mounting data have suggested that LCSCs develop through a multistep process associated with RNAs, genes, proteins, pathways, factors, autophagy, the microenvironment and the networks between them. Thus, a better understanding of the molecular mechanisms underlying HCC initiation and progression is a pressing requirement. Additional studies are urgently necessary to facilitate exploration of new therapeutic targets and effective treatment strategies. Through classification of the studies on LCSC targeting published in the past 5 years, we found that most of the studies focused on ncRNAs (especially the miRNAs and lncRNAs), oncogenes, oncoproteins and the crosstalk between their upstream/downstream genes and molecular pathways.

miRNA is a major class of non-protein-coding transcripts that instead function in posttranscriptional regulation of genes. Several miRNAs can enhance LCSC features, and opposite effects can be found with other miRNAs. lncRNAs regulate gene expression through different ways, such as protein and miRNAs networks. In this review, we observed that a number of miRNAs and lncRNAs might serve as novel markers or provide potential therapeutic targets in LCSCs. Dysregulation of miRNAs or lncRNAs could be used to identify and characterize LCSCs based on their interaction with pivotal signaling pathways, focusing on the Wnt/β-Catenin signaling pathway (such as miR-1246, miR-429, Lnc-β-Catm, lncTCF7, and CUDR), IL6/JAK2/STAT3 signaling pathway (such as miR-500a-3p, miR-589-5p, DLX6-AS1, and Lnc-DILC), PI3K/Akt/Bad signaling pathway (such as miR302a/d, miR-1305, miR24-2, miR-25, and lncRNA-HULC) and certain genes, including OCT4 (such as miR-1246 and miR-429) and Nanog (such as miR24-2), as well as on cell surface proteins or cellular prognostic markers that have been identified to be characteristic of LCSCs, such as EpCAM (miR26b-5p and miR-429).

Genes support the basic structure and properties of life through their genetic effects. In this review, according to **Supplementary Table 1**, which presents impact factors, we found that the oncogenes and oncoproteins reported by high-impact factor studies to target LCSCs are primarily located in the nucleus and cytoplasm. Similarly, we found that many oncogenes and oncoproteins are novel potential LCSC markers located in the cell membrane or are subsecretory types. Moreover, some of the molecular mechanisms of the oncogenes or oncoproteins that target LCSCs are the same and involve several key pathways, including the Wnt/β-catenin signaling pathway (such as Sox9, Shp2, MYCN, ZFX, GLS1, Tg737, KLF8, and Ring1), Notch signaling pathway (such as iNOS and CD44s), PI3K/Akt/c-Myc pathway (such as HOXB7, Cygb, and ITGA7) and STAT3 pathway (such as AQP3). The target genes include Nanog (such as Sox9, NUMB, TARBP2, RACK1, and FOXM1) and OCT4 (such as Sox9, ZIC2, FOXM1, and BORIS), along with LCSC biomarkers (such as CD133, CD44, and EpCAM).

From the above observations, in addition to LCSC surface biomarkers, we emphasize the role of three signaling pathways and two genes that influence LCSCs. The first is the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway has been identified as one of the most frequent participants in CSCs (Fodde and Brabletz, 2007). Dramatically, the Wnt/β-catenin signaling pathway, which involves translocation of β-catenin to the nucleus, is heavily implicated in LCSCs (Yamashita et al., 2007). Moreover, the final nuclear transfer can induce transcription of prominent targets, such as c-Myc (He et al., 1998) and CD44 (Wielenga et al., 1999). CD44 has also been identified

as a biomarker of LCSCs (Zhu et al., 2010). In addition, EpCAM is a direct transcriptional target of the Wnt/β-catenin signaling pathway in HCCs (Yamashita et al., 2007).

The second signaling pathway is the PI3K/Akt/c-Myc pathway. PI3K-Akt has been shown to promote cancer stemness in various cancer types (Hambardzumyan et al., 2008; Bleau et al., 2009). Elevated phosphatidylinositol 3,4,5-trisphosphate (PIP)3 levels lead to activation of multiple kinases, including phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates downstream targets, such as Akt. Activated Akt phosphorylates numerous substrates to regulate vital cellular processes, including tuberous sclerosis complex 2 (TSC2), NF-κB and GSK3β (Vanhaesebroeck et al., 2010). Furthermore, the PI3K-Akt pathway has been reported to augment the expression of c-Myc (Tsai et al., 2012; Zhang H.F. et al., 2016). Interestingly, one study demonstrated synergistic interactions of CD44 and TGF-β1 in EMT induction via the Akt/GSK-3β/β-catenin pathway in HCCs (Park et al., 2016). Here, we found that c-Myc is a coactive gene in the Wnt/β-catenin signaling pathway and PI3K/Akt signaling pathway. Interestingly, the proto-oncogene Myc is the frequent event in many cancers (Soucek et al., 2008). Myc can be activated via Wnt/β-catenin, PI3K/Akt, MAPK/extracellular signal-regulated kinase (ERK) and Hedgehog. Mechanically, the activated Myc gene affects target genes mediation including chromatin remodeling and DNA-methylation (Sridharan et al., 2009).

The third signaling pathway is the IL6/JAK2/STAT3 signaling pathway. IL-6 produced by tumor-associated macrophages (TAMs) can activate the STAT3 signaling pathway to promote $CD44^+$ LCSCs (Wan et al., 2014). Therefore, an IL-6 receptor blocking antibody (such as tocilizumab) is a novel therapeutic strategy for targeting LCSCs. Simultaneously, it has been demonstrated that targeting of the TGF-β pathway using indirect modulation of IL6/STAT3 appears to effectively eradicate LCSC features (Lin et al., 2009).

OCT4, which belongs to the POU family, is the most important stem cell factor and is considered the master regulator in the maintenance of stem cell potency (Nichols et al., 1998). Active OCT4 can directly regulate two downstream stem cell regulator genes, Nanog and SOX2, promoting LCSC-like phenotypes (Babaie et al., 2007). Many studies have identified that there is a correlation between OCT4 and LCSCs (Murakami et al., 2015). Nanog has been proposed as an important regulator modulating the phenotype of CSCs in various of cancer types (Shan et al., 2012; Chen et al., 2016). Furthermore, one study has reported that overexpression of CD24 is accompanied by increased STAT3 and Src activities (Bretz et al., 2012). Interestingly, STAT3-mediated Nanog expression can regulate self-renewal and tumor initiation in $CD24^+$ LCSCs (Lee et al., 2011).

Altogether, non-coding RNAs, genes, and signaling pathways form a network that affects the characteristics of LCSCs. Targeting LCSCs via ncRNAs, oncogenes, oncoproteins or signaling pathways holds promise for preventing disease relapse. In addition, some small molecular agents have been studied extensively. However, there is still no available US FDA-approved drug that is likely to be clinically effective for HCC. It is now clear that all RNAs, genes, proteins and signaling pathways function as a coordinated network rather than operating in isolation. Thus, we should find a key node in the LCSC network. In this review, we summarize three pathways: the Wnt/β-catenin pathway, PI3K/Akt pathway, and IL6/JAK2/STAT3 pathway and their targeting gene c-Myc. Furthermore, we conclude that two important genes are OCT4 and Nanog. They play a pivotal role in LCSC regulation and HCC prognosis. There is a potential opportunity to achieve great therapeutic effects by targeting the above signaling pathways or genes in LCSCs. However, their dual oncogenic and biological functions indicate that targeting should be conducted with caution.

AUTHOR CONTRIBUTIONS

YZ conceived and designed the work and approved the final version. JL acquired the data and wrote the manuscript. Both authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Identifying 8-mRNAsi Based Signature for Predicting Survival in Patients With Head and Neck Squamous Cell Carcinoma via Machine Learning

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Cancer stem cells (CSCs) have been characterized by several exclusive features that include differentiation, self-renew, and homeostatic control, which allows tumor maintenance and spread. Recurrence and therapeutic resistance of head and neck squamous cell carcinomas (HNSCC) have been identified to be attributed to CSCs. However, the biomarkers led to the development of HNSCC stem cells remain less defined. In this study, we quantified cancer stemness by mRNA expression-based stemness index (mRNAsi), and found that mRNAsi indices were higher in HNSCC tissues than that in normal tissue. A significantly higher mRNAsi was observed in HPV positive patients than HPV negative patients, as well as in male patients than in female patients. The 8-mRNAsi signature was identified from the genes in two modules which were mostly related to mRNAsi screened by weighted gene co-expression network analysis. In this prognostic signatures, high expression of *RGS16, LYVE1, hnRNPC, ANP32A,* and *AIMP1* focus in promoting cell proliferation and tumor progression. While *ZNF66, PIK3R3,* and *MAP2K7* are associated with a low risk of death. The riskscore of eight signatures have a powerful capacity for 1-, 3-, 5-year of overall survival prediction (5-year AUC 0.77, 95% CI 0.69–0.85). These findings based on stemness indices may provide a novel understanding of target therapy for suppressing HNSCC stem cells.

Keywords: cancer cell stemness indices, head and neck squamous cell carcinomas, The Cancer Genome Atlas, weighted gene co-expression network analysis, predictive models

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INTRODUCTION

Head and neck cancers are a collection of malignancies that arise from the upper aerodigestive tract, salivary glands and thyroid (Cramer et al., 2019). Head and neck squamous cell carcinomas (HNSCC) account for 90% of head and neck cancers and are mainly derived from the oral cavity, oropharynx, hypopharynx, and larynx (Wyss et al., 2013). The main reasons associated with their occurrence are tobacco and alcohol use, however, increased HNSCC cases with human papillomavirus (HPV) have highlighted the role of high-risk HPV in the pathology of HNSCC (Chaturvedi et al., 2011). Worldwide, around 430,000 patients die due to its high mortality annually, where its 5-year survival rate is about 40–50%, though patients with the advanced disease only have a 34.9% survival rate (Leemans et al., 2011). Hence, it is critical to explore the mechanism regarding this malignancy, which may aid in diagnosing early HNSCC and predicting clinical outcomes.

Stem cells are known to be a cell subset having the ability to self-renew and differentiate, which has been found in most human tissues (Blanpain et al., 2004). Due to strides in cancer research, cancer cells are generally considered to have the propensity to initiate, spread and metastasize. Several studies based on multiple tumors showed that a small subpopulation of undifferentiated cells that strikingly resemble stem cells within the tumor could trigger cancers. Therefore, these cells were aptly named cancer stem cells (CSCs; Reya et al., 2001). Cancer stem cells are present in bulk tumors of HNSCC and gave rise to new tumors in immunodeficient mice (Prince et al., 2007; Okamoto et al., 2009), which may elucidate how residual stem cells cause tumor recurrence and regrowth in patients following treatment. To further clarify CSCs, researchers fused artificial intelligence and deep learning methods further to explore the features of stem cells in tumors. Malta et al. (2018) generated stemness indices for evaluating the degree of oncogenic dedifferentiation using a one-class logistic regression machine learning algorithm (OCLR), which may define signatures to quantify stemness. Accordingly, they extracted transcriptomic and epigenetic feature sets from non-transformed pluripotent stem cells and their differentiated progeny, eventually obtaining the two stemness indices, mDNAsi and mRNA expression-based stemness index (mRNAsi).

This study attempts to generate the stem cell-associated indices by taking advantage of both the Progenitor Cell Biology Consortium (PCBC) and The Cancer Genome Atlas (TCGA) databases, which analyzed and quantified cancer stemness in the HNSCC cohort and acquired their mRNAsi scores. Using weighted gene co-expression network analysis (WGCNA), gene modules were constructed that are closely related to the stem index. Eight mRNAsi based signatures were selected from two of these gene modules, and a risk model based on eight mRNAsi signatures was conducted to predict the prognostic risk in HNSCC patients. Finally, a functional analysis was carried out to determine the molecular mechanism's stemness regarding the prognosis of HNSCC patients.

MATERIALS AND METHODS

Data Collection and Pre-processing

The CSC samples were downloaded from the PCBC R package synapser (v 0.6.61). Moreover, the raw data of gene expression and related clinical information of HNSCC patients were downloaded from the TCGA website, which included 546 RNA-Seq expression data. Additionally, 97 cases of GSE41613 data were downloaded from the Gene Expression Omnibus (GEO) website. The RNA-Seq data from TCGA-HNSCC were pre-processed as follows. Samples with expression profile information were retained, changing the Ensemble ID to Gene Symbol, while only leaving protein-coding genes. Next, the expression data of primary solid tumors and solid normal tissue samples were left. Afterward, the expression of multiple genes was chosen as the median. Finally, the overall survival (OS) data used for the survival analysis removed samples with a survival time of less than 30 days. GSE41613 data was also pre-processed, and the samples kept their expression profile information. Moreover, the unit of survival information of the sample was converted to days, and the probe was changed to the Gene Symbol. The probes which were related to several genes were deleted, and the expression of multiple genes was chosen as the median. As the TCGA data, the OS data used for the survival analysis removed samples with a survival time less than 30 days. All data from these two databases after pre-processing are shown in **Table 1**.

CSCs-Related Clinical Characteristics of HNSCC

The expression data of pluripotent stem cells (ESC and iPSC) from the PCBC database were analyzed, and the OCLR algorithm was utilized to predict mRNAsi. The Kruskal-Wallis test then compared the mRNAsi of normal tissue and tumor tissue or different clinical characteristics.

Weighted Gene Co-expression Network Analysis

Module Establishment

The WGCNA co-expression algorithm was utilized to acquire the co-expressed genes and co-expression modules according to the expression profiles of these genes. According to the 500 HNSCC expression data from the TCGA database, the expression profiles of the protein-coding genes were extracted. A co-expression network was constructed using WGCNA in the R package based on the TCGA datasets. A Pearson correlation matrix was built to calculate the distance of each gene.

In this study, a soft threshold of nine was selected to screen the co-expression modules. To ensure the constructed co-expression network approached the scale-free distribution, $\beta = 9$ was chosen. Next, the expression matrix was changed to the adjacency matrix, after which the adjacency matrix was converted into a topological overlap matrix (TOM). Average linkage hierarchical clustering was used to cluster genes based on TOM, and the minimum genome number of the gene dendrogram was 40.

TABLE 1 | Clinical information of TCGA-HNSCC and GSE41613.

Clinical features	TCGA-STAD	GSE41613
Type		
Normal	44	0
Tumor	500	97
OS		
0	280	46
1	211	50
OS time (mean)		
0	1047.261	1997.23
1	767.1185	730.65
T Stage		
T1	34	
T2	143	
T3	132	
T4	180	
TX	11	
N Stage		
N0	241	
N1	81	
N2	152	
N3	7	
NX	19	
M Stage		
M0	475	
M1	5	
MX	20	
Stage		
I	25	
II	81	
III	90	
IV	304	
Grade		
G1	61	
G2	299	
G3	119	
G4	2	
GX	19	
Gender		
Male	367	
Female	133	
Age		
≤60	244	
>60	255	
Unknown	1	
Alcohol		
Yes	332	
No	157	
Unknown	11	
HPV Status		
Negative	64	
Positive	19	
Unknown	417	
Tobacco		
1	111	
2	170	
3	72	
4	135	

Identifying mRNAsi Modules

After determining the genetic modules, the module eigengenes of each module, in turn, was calculated, and the modules were then clustered, resulting in 20 differently related modules. The relationship between each module and different clinical characteristics was also analyzed. The most positive correlation was with the blue module, while the most negative correlation was with the yellow module.

Functional Annotation: Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analyses

The WebGestaltR (v0.4.2) R package was adopted for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and Gene Ontology (GO) functional annotation to investigate the biological functions of key modules and genes. In our study, we identify over-represented GO terms in three different categories: biological processes, molecular function and cellular component, and over-represented KEGG pathway terms. Furthermore, FDR < 0.05 was considered to be statistically significant.

Construction and Analysis of the Risk Prognosis Model

The 491 TCGA samples were random as a 0.5:0.5 ratio divided into the training and test sets as previously described (Wang et al., 2020). Then, using the training set samples, the genes were further identified using a univariate Cox regression analysis of the survival coxph function package in the R language, where $p < 0.01$ was used as the threshold to optimize the data. Least absolute shrinkage and selection operator (Lasso) regression analysis was then used to reduce the number of genes, resulting in 17 genes. Next, the Akaike information criterion (AIC) was utilized to optimize the data, and a total of eight genes were finally identified for further use. The corresponding eight genes were used to build a prognostic risk score model.

The formula of the risk score model is described as:

$$\begin{aligned} RiskScore \;=\; & 0.20799^{\times} RGS16 + 0.2492^{\times} LYVE1 - 0.8828^{\times} \\ & MAP2K7 - 0.2654^{\times} PIK3R3 - 0.5666^{\times} ZNF66 \\ & +0.6486^{\times} hnRNPC + 0.7821^{\times} ANP32A + 0.5284^{\times} AIMP1 \end{aligned}$$

We used TCGA training set to test whether the gene markers were independent prognostic factors, and multivariate Cox regression analysis was used. Receiver operating characteristic (ROC) curve was depicted using the timeROC package in R. Samples in H (High) set had a significantly higher score compared to those in the L (Low) set, where "0" was used to divide the two sets. A Kaplan–Meier (KM) curve was drawn. Significance was defined as $P < 0.05$.

Module and Clinical Trait Association Prognosis Analysis

The relationship between different clinical traits and OS time survival curves were plotted from the KM estimates. For the 8-mRNAsi based signature associations, some groups were clearly distinct to high or low expression groups.

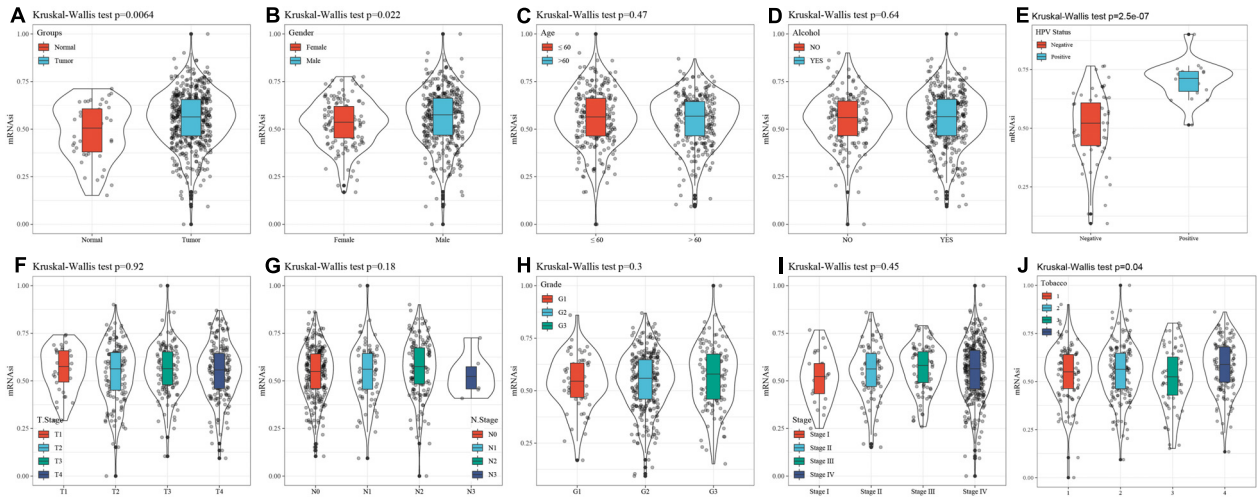

FIGURE 1 | Correlation between mRNAsi and clinical characteristics in HNSCC. **(A)** The different expressions of mRNAsi between normal and tumor samples. **(B)** The different expressions of mRNAsi between gender-specific samples. **(C)** The different expressions of mRNAsi between different age samples. **(D)** The different expressions of mRNAsi between drinking alcohol status samples. **(E)** The different expressions of mRNAsi between different HPV status samples. **(F)**The different expressions of mRNAsi between different T staging. **(G)** The different expressions of mRNAsi between different N staging samples. **(H)** The different expressions of mRNAsi between different Grade grading samples. **(I)** The different expressions of mRNAsi between different Stage staging samples. **(J)** The different expressions of mRNAsi between smoking status samples.

Gene Set Enrichment Analysis and Gene Set Variation Analysis (GSVA)

The R package was employed to perform the gene set enrichment analysis (GSEA) analysis of the key genes. Meanwhile, the "gene set variation analysis (GSVA)" R package was used to find the most associated pathways with the 8-mRNAsi based signature. Based on the different functions according to the score of each sample, the correlation between these functions and risk was further calculated, and the most associated pathways were identified.

Cell Culture

Human HNSCC cell lines FaDu, JHU011 and HN8 were kindly provided by the Xiangya Hospital of Central South University. FaDu cell was cultured in MEM medium (Sigma, MO, United States), JHU011cell was cultured in RPMI-1640 and HN8 cell was cultured in DMEM medium (Sigma, MO, United States). All the medium were supplemented with 10% FBS and 1% penicillin/streptomycin, maintained on plastic plates and incubated at 37°C with 5% CO_2.

RT-qPCR Assay

According to the manufacturer's protocol, total RNA of cells was extracted using TRIzol (Life Technologies, Carlsbad, CA, United States). After cDNA synthesis (All-in-One First-Strand cDNA Synthesis kit, GeneCopoeia Inc, Santa Cruz, CA, United States), the quantitative real-time polymerase chain reaction (qPCR) experiment was carried out using All-in-One qPCR Mix (GeneCopoeia Inc, United States) on ABI 7500HT System (Applied Biosystems, Foster City, CA, United States) using primers were described as **Supplementary Table 1**. The PCR detailed reaction conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. GAPDH was used as the internal control in this study. The relative expression of target genes was controlled to GAPDH and $2^{-\Delta\Delta CT}$ method was calculated to evaluate relative mRNA levels. All the experiments were run in triplicate.

RESULTS

Relationship Between mRNAsi and Clinical Characteristics in Head and Neck Cancer

mRNA expression-based stemness index is a particular stemness index, which is considered to be a biomarker in CSCs. 78 cases of expression data from pluripotent stem cells were downloaded from PCBC. Here, mRNAsi in HNSCC tissues was significantly higher than that of normal tissues (p = 0.0064) (**Figure 1A**). Moreover, to discover the correlation of mRNAsi with the corresponding clinical characteristics, the downloaded information contains the gender, age, disease stage, tumor stage classification, node stage classification, clinical grade, HPV status, smoking status, and alcohol status. The result of the Kruskal–Wallis test showed that male patients had a significantly higher mRNAsi than female patients (p = 0.022) (**Figure 1B**). Meanwhile, there was a difference in mRNAsi in the smoking status group (p = 0.04) (**Figure 1J**). And the result of Kruskal–Wallis test indicated that HPV positive patients had a significantly higher mRNAsi than HPV negative patients (p = 2.5e-07) (**Figure 1E**). In terms of age, alcohol status, tumor classification, node classification, and disease stage, no significant difference in the mRNAsi was present among the tumor tissues (**Figure 1**).

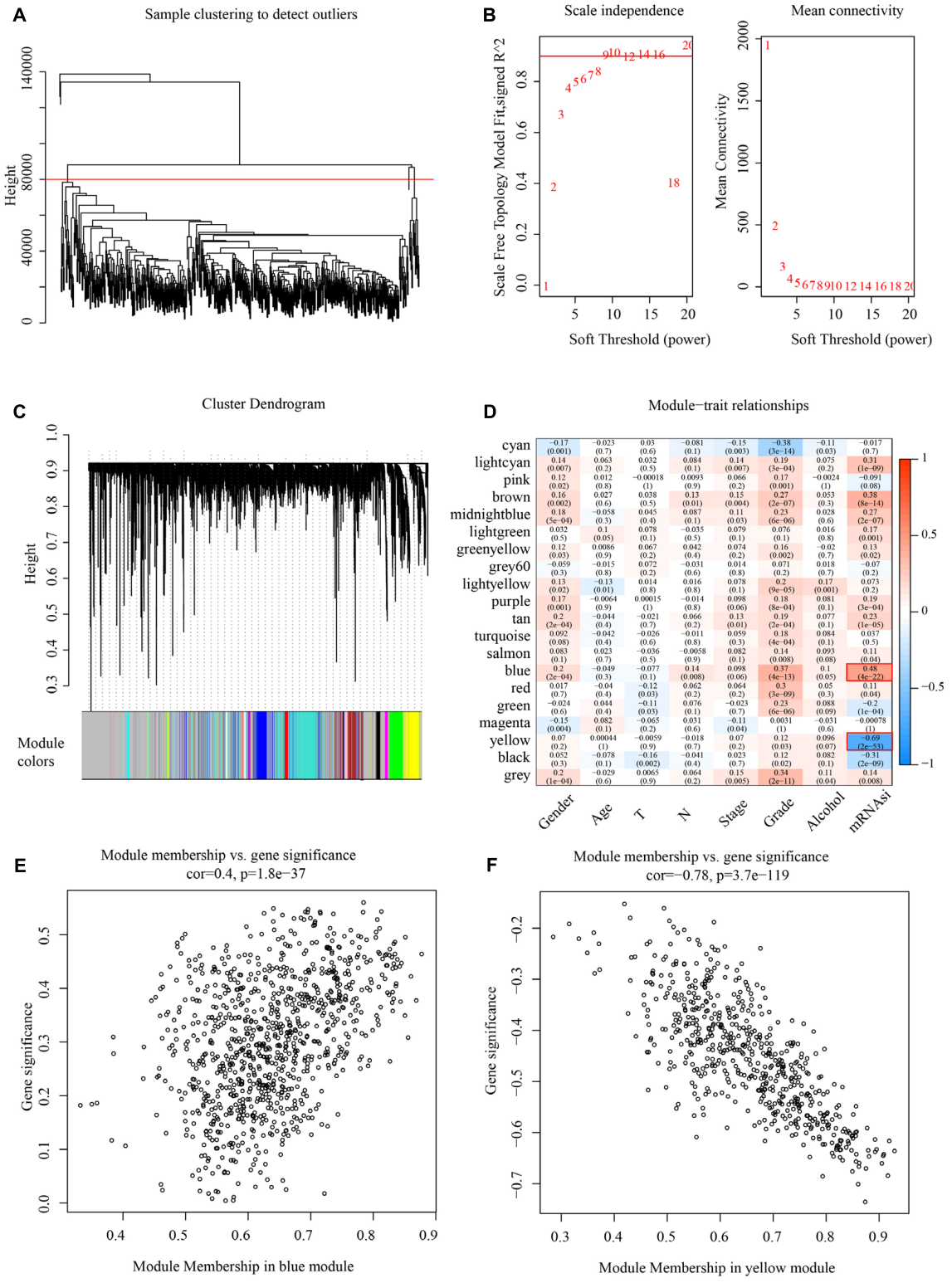

FIGURE 2 | Identify mRNAsi basedgene modules in HNSCC. **(A)** Cluster analysis. **(B)** Analysis of network topology for various soft-thresholding powers. **(C)** Gene dendrogram and module colors. **(D)** Results of correlation between twenty modules and each clinical phenotype. **(E)** Correlation of blue modules and genes. **(F)** Correlation of yellow modules and genes.

WGCNA: Head and Neck Cancer Stem Cell Index and Gene Expression Analysis

Weighted gene co-expression network analysis analyzes the molecular interactions according to the co-expression network (Tian et al., 2018). Here, the expression profiles of protein-coding genes were selected according to 500 gene expression profiles of head and neck cancer from the TCGA database. Hierarchical clustering was then used to analyze sample clustering (**Figure 2A**). To this effect, $\beta = 9$ (**Figure 2B**) was chosen as a soft scale to ensure a scale-free network, culminating with 20 gene modules for further analysis (**Figure 2C**).

The correlation of mRNAsi with clinical factors like gender, age, TNM classification, and clinical stage was examined, as shown in **Figure 2D**, where the most significant positive correlation module with mRNAsi is the blue module, and the most negative correlation module with mRNAsi is the yellow module. And these two modules contain 1518 genes, and all the genes are shown in **Supplementary Table 2**. The module membership in the blue module was shown in **Figure 2E** and the module membership in the yellow module was shown in **Figure 2F**.

Gene Modules Functional Annotation Analysis

This study employed GO and KEGG for the functional enrichment analysis of the blue and yellow modules. For the blue module, the study results show that all the top 10 significantly enriched factors with GO, Biological process (BP), Cellular component (CC), and KEGG pathways were obtained, as presented in **Supplementary Figure 1**. Notably, p53 signaling pathway, DNA replication and cell cycle are related to cancer, as we found in KEGG pathway analysis. Then for the yellow module, we can also get the results that the top 10 significantly enriched factors with GO, BP, CC, and KEGG pathways were presented in **Supplementary Figure 2**. Among all enriched KEGG pathways, the PI3K-Akt signaling pathway, MAPK signaling pathway and ECM–receptor interaction are related to cancer.

Construct a Gene Prognostic Risk Model Based on mRNAsi

mRNAsi-Related Gene Prognostic Risk Models

The 491 samples were selected from TCGA and were randomly divided into training sets and test sets (**Table 2**). Additionally, 246 patients from the training set were used in the following survival analysis. According to the univariate Cox regression model and Lasso cox regression model, 17 genes were acquired for subsequent analysis. Afterward, AIC was used to optimize the data, and a total of eight genes were finally identified to analyze: *RGS16, LYVE1, MAP2K7, PIK3R3, ZNF66, hnRNPC, ANP32A,* and *AIMP1*.

The KM curves showed that, except for LYVE1 and PIK3R3, the remaining six genes had significantly divided the samples from the training set into two groups, high risk groups and low risk groups (**Figure 3**).

The riskscore of the training set was calculated according to the expression level of each sample, and the distribution of RS is shown in **Figure 4A**. The OS time of patients with high RS was found to be significantly lower than ones with low RS. *RGS16, LYVE1, hnRNPC, ANP32A*, and *AIMP1* with high expression represent risk factors. Moreover, *ZNF66, PIK3R3*, and *MAP2K7* attained the opposite result, making them protective factors. We further applied the timeROC package to analyze the prognosis of 1-, 3-, and 5-year survival rates. Accordingly, the model was found to exhibit that 1-year AUC 0.74, 95% CI 0.66–0.81, 3-year AUC = 0.78, 95% CI 0.72–0.84, and 5-year AUC 0.77 95% CI 0.69–0.85 (**Figure 4B**).

Additionally, riskscore was utilized to make the zscore, where all zscore samples greater than zero were included in the high risk group, while the rest of the samples smaller than zero were divided into the low risk group. Finally, 118 high risk samples and 128 low risk samples were obtained, the survival time between high and low risk samples was significantly ($p < 0.0001$; **Figure 4C**).

TABLE 2 | Clinical information statistics for TCGA train set and test set.

Clinical Features	TCGA-train	TCGA-test	*P*
OS			
0	146	134	0.3417
1	100	111	
T Stage			
T1	18	15	0.4751
T2	64	77	
T3	72	58	
T4	86	91	
TX	6	4	
N Stage			
N0	112	125	0.4721
N1	40	39	
N2	82	68	
N3	2	5	
NX	10	8	
M Stage			
M0	233	234	0.396
M1	4	1	
MX	9	10	
Stage			
I	10	15	0.3178
II	46	34	
III	41	49	
IV	149	147	
Grade			
G1	30	30	0.5258
G2	145	148	
G3	63	54	
G4	0	2	
GX	8	11	
Gender			
Male	180	181	0.9401
Female	66	64	
Age			
≤60	129	113	0.1904
>60	117	132	

Risk Model Verification

To verify the robustness of the 8-mRNAsi based signature model, we calculated a riskscore in TCGA test set and an external dataset (GSE41613). Regarding the TCGA test dataset (**Supplementary**

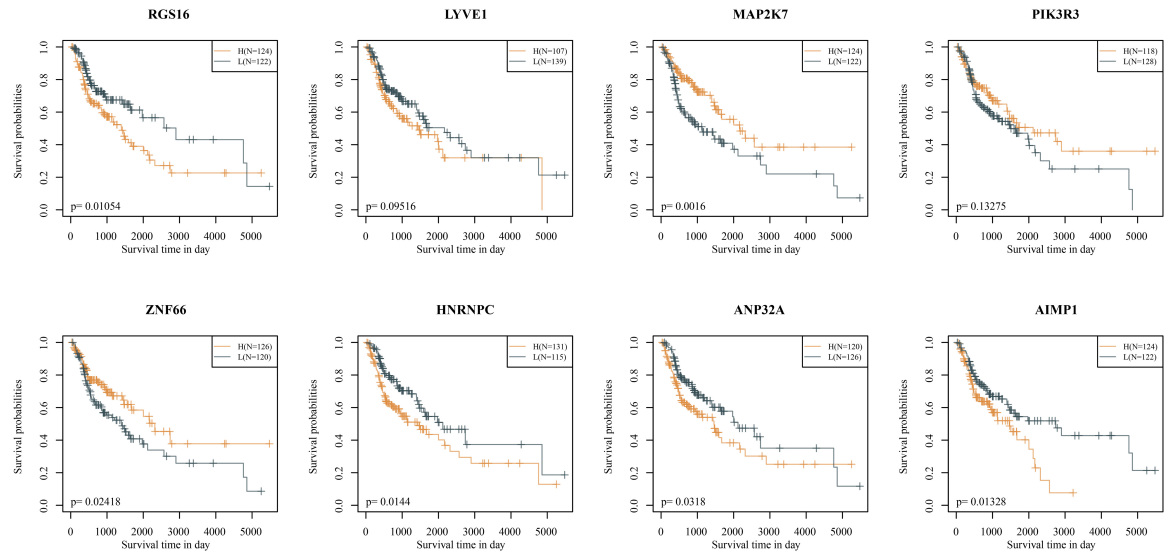

FIGURE 3 | Kaplan–Meier curves of eight signatures (in the TCGA training set).

Figure 3), we found the same results as the training set was yielded for, where ROC analysis showed that the 5-year AUC was up to 0.70. The survival time between high and low risk samples was significantly different ($p < 0.0001$). For the GSE41613 database (**Supplementary Figure 4**), ROC analysis showed that the average 1-, 3-, and 5-year AUC for the 8-mRNAsi based signature was close to 0.67, 95%, the relationship between the expression of the eight genes and risk score is also consistent with the training set.

Risk Model and Analysis of Clinical Features of Prognosis

A series of KM curves graphs were made to analyze the prognosis. As shown in **Figure 5**, patients with HNSCC were analyzed according to nine clinical features (tumor classification, Node classification, disease stage, grade, gender, age, alcohol status HPV status and smoking status). The meaning of the four different smoking status in **Figure 5I** was as follows: Lifelong Non-smoker (less than 100 cigarettes smoked in Lifetime) = Tabacco1; Current smoker (includes daily smokers and non-daily smokers or occasional smokers) = Tabacco2; Current reformed smoker for > 15 years (greater than 15 years) = Tabacco3; Current reformed smoker for ≤15 years (less than or equal to 15 years) = Tabacco4. The results showed that only the stage group and HPV status were related to OS time ($p < 0.05$) (**Figures 5C,H**), and the prognosis was worse with increasing disease stage and with HPV negative patients.

To further explore the influence of clinical features on the OS of the 8-mRNAsi based signature, all clinical features were stratified. Then, every stratified feature was divided into high-risk and low-risk groups. As shown in **Figure 6**, the 8-mRNAsi based signature acted as a risk factor for patients with different clinical characteristics.

We performed univariable and multivariable Cox regression analysis to evaluate the 8-mRNAsi based signature related HR, 95% CI of HR, *P*-value. Clinical characteristics, including alcohol status, age, tumor stage classification, node stage classification, pathological grade, disease stage, and riskscore, were systematically analyzed. Our results from the TCGA database showed that riskscore from either univariable (HR = 1.913, 95% CI 1.642-2.228, p = 2.0E-16) or multivariable Cox regression analysis(*HR* = 1.872, 95% CI 1.613-2.173, p = 2.0E-16) are significantly correlated to survival (**Table 3**). And the same result can be obtained in node stage classification and disease stage. In node stage classification group, univariable (HR = 1.205, 95% CI 1.045-1.389, p = 0.010) or multivariable Cox regression analysis (HR = 1.195, 95% CI 1.015-1.406, p = 0.032) are correlated to survival (**Table 3**). Meanwhile, in disease stage group, univariable (HR = 1.345, 95% CI 1.138-1.589, p = 5.0E-04) or multivariable Cox regression analysis (HR = 1.310, 95% CI 1.056-1.625, p = 0.014) are significantly correlated to survival (**Table 3**).

Relationship Between Riskscore and Signaling Pathway

To analyze the KEGG functional enrichment score for each sample in the training set, GSVA was utilized in the R software package for the GSEA analysis.

The scores were calculated from each sample with different functions to acquire the ssGSEA score of each function corresponding to each sample, where the relationship between functions and riskscore was further verified. The function with a correlation greater than 0.25 was selected, as shown in **Figure 7A**.

Here, 13 cases had a positively correlated with the sample risk score, while two had a negative correlation. The most related ten KEGG pathways were chosen and were clustered based on their enrichment score (**Figure 7B**). Accordingly, among all pathways, the riskscore rises as KEGG_COMPLEMENT_AND_COAGULATION_CASCADES, KEGG_NITROGEN_METABOLISM, and KEGG_TGF_BETA_SIGNALING_PATHWAY rises, and for KEGG_REGULATION_

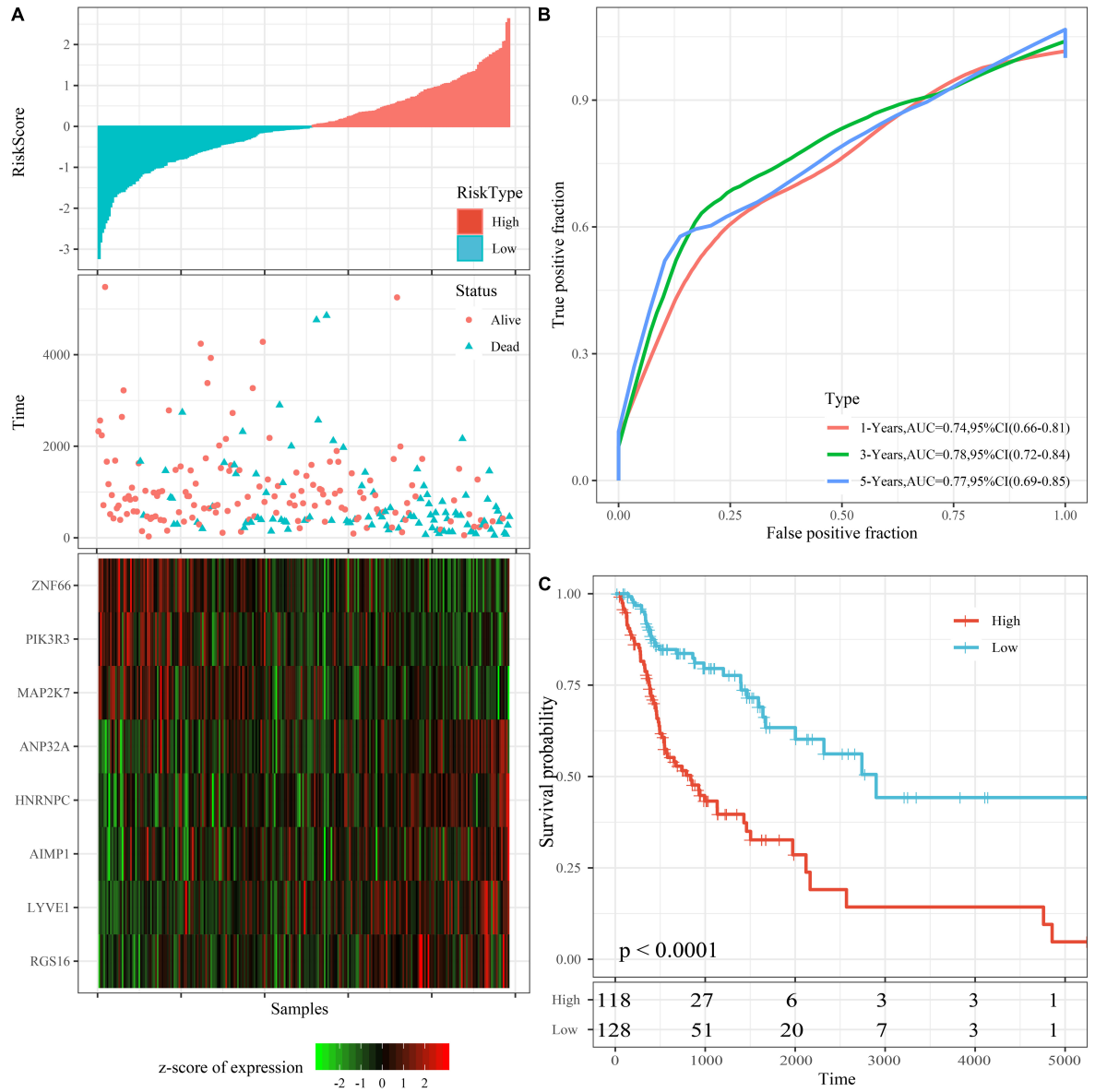

FIGURE 4 | Performance of the 8-mRNAsi based signature model with TCGA training set. **(A)** Survival time, survival status and 8-genes expression of Riskscore in the training set. **(B)** ROC Curve and AUC of 8-gene signature Classification. **(C)** The KM survival curve distribution of 8-gene signature in the training set.

OF_AUTOPHAGY, KEGG_TASTE_TRANSDUCTION, KEGG _ABC_TRANSPORTERS, the riskscore decreases as they increase.

Expression Level of Eight mRNAsi in HNSCC Cell Lines as Detected by a RT-qPCR Assay

We tested the expression levels of eight mRNAsi in FaDu, JHU011, and HN8 cell lines by a RT-qPCR assay. The results showed that *RGS16, LYVE1, hnRNPC, ANP32A and A1MP1* were highly expressed in all cell lines. And *ZNF66, PIK3R3 and MAP2K7* were lowly expressed in three cell lines (**Figure 8**).

DISCUSSION

Many advanced therapeutic and diagnostic methods have been carried out in modern HNSCC treatment, though their effects remain inadequate as the oncologists anticipated. CSCs, due to their strong self-renewal ability, are thought to play an essential role associated with invasive potential, tumor growth and therapeutic resistance in response to the development of HNSCC (Peitzsch et al., 2019). Therefore, identifying therapeutic targets for CSCs would be significant in anti-cancer treatment. As a type of heterogeneous malignancy, a molecular analysis of HNSCC tissues demonstrates high intratumoral heterogeneity determined by clonal evolution of the CSCs populations (Yang et al., 2020).

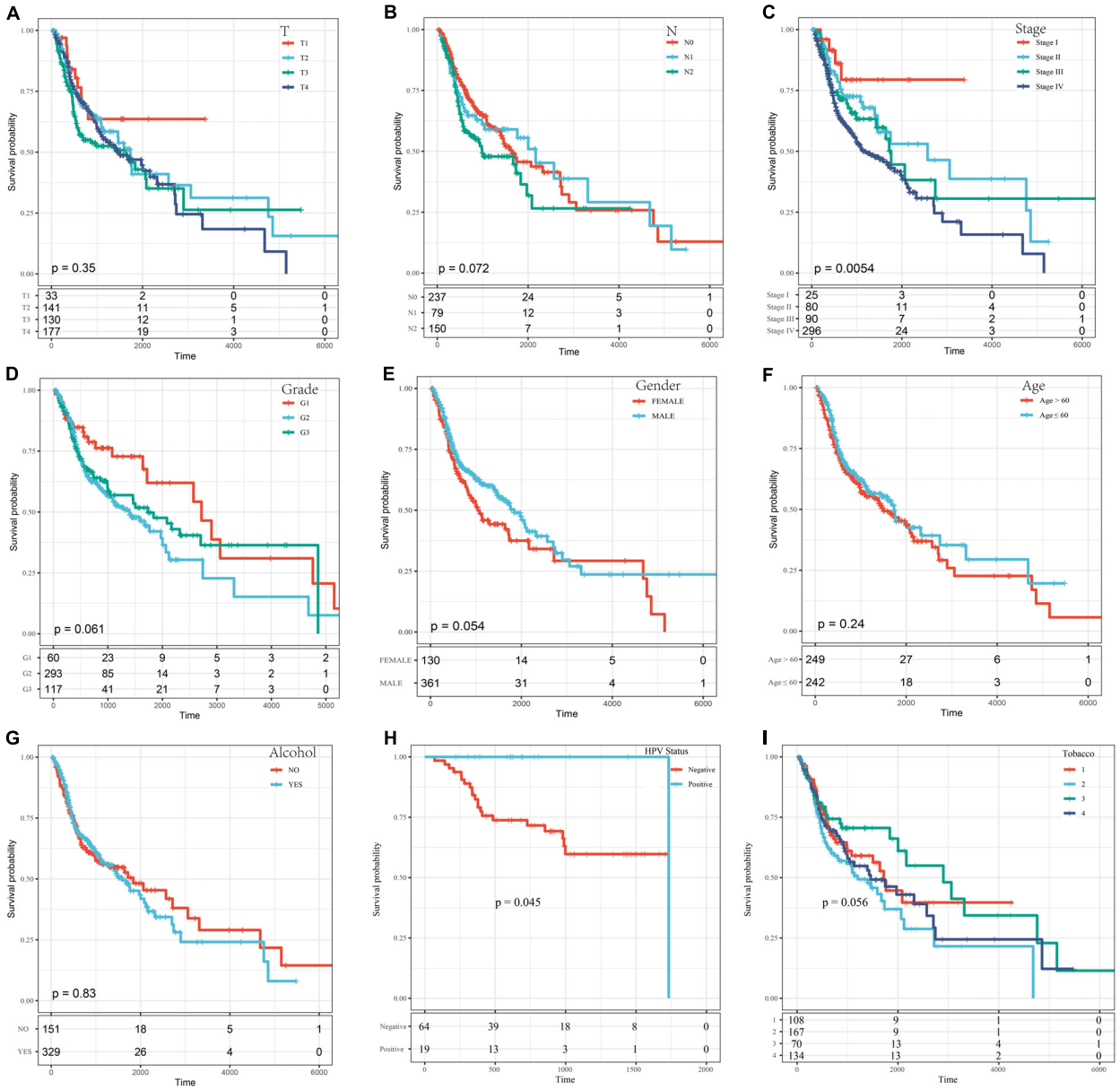

FIGURE 5 | The KM curves of different clinical characteristics. **(A)** KM curves of different tumor classifications. **(B)** KM curves of different node classifications. **(C)** KM curves of different disease stages. **(D)** KM curves of different cancer grades. **(E)** KM curves of different genders. **(F)** KM curves of young (age ≤ 60) and elderly (age > 60) ages. **(G)** KM curves of different alcohol status. **(H)** KM curves of different HPV status. **(I)** KM curves of different smoking status.

In the present study, the correlation of mRNAsi indices between normal tissues and HNSCC tissues were presented based on the OCLR machine-learning algorithm (Malta et al., 2018). In line with previous studies regarding other cancers (Malta et al., 2018; Lian et al., 2019), a significantly higher level of mRNAsi was observed in HNSCC tissues compared to that in normal tissues. By comparing the mRNAsi with the clinical characteristics, which revealed that mRNAsi had a significant rise in HPV positive patients, and that male patients had a higher mRNAsi indices than female patients. This result may suggest a potential correlation of HPV status with CSCs. One study of four HPV negative HNSCC cell lines were infected with HPV genome, which resulted in tumor cells have increased growth and self-renewal capacity (Lee et al., 2015). Zhang reported a study of six oropharyngeal HNSCC tumor specimens, where HPV positive tumors had a higher proportion of CSCs compared to HPV negative tumors in six specimens of HNSCC, which was attributed to p53 inactivation by HPV (Zhang et al., 2014). P53 is an essential target of HPV-E6/E7 proteins that bind to p53 resulting in the deregulation of p53 and causing a more proliferative state (Jin and Xu, 2015). Conversely, Tang determined that CSCs population are not affected by HPV in

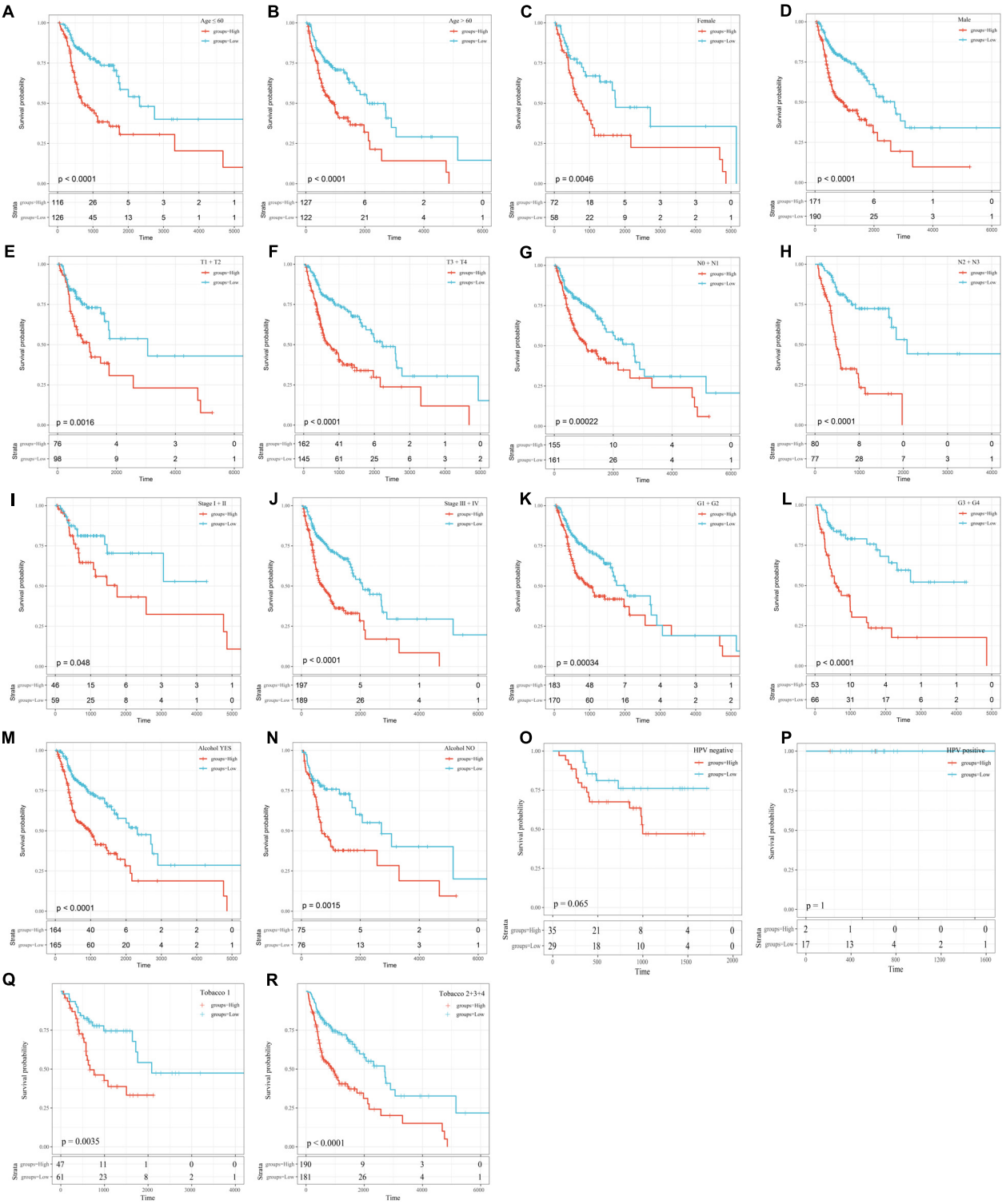

FIGURE 6 | KM curves showing the OS of each subgroup of HNSCC patients with high or low riskscores. **(A)** KM curves of high and low risk samples in the young (age ≤ 60). **(B)** KM curves of high and low risk samples in the elderly (age > 60). **(C)** KM curves of Female samples. **(D)** KM curves of Male samples. **(E)** T1+T2 KM curves of high and low risk samples. **(F)** T3+T4 KM curves of high and low risk samples. **(G)** N0+N1 KM curves of high and low risk samples. **(H)** N2+N3 KM curves of high and low risk samples. **(I)** Stage I+II KM curves of high and low risk samples. **(J)** Stage III+IV KM curves of high and low risk samples. **(K)** G1+G2 KM curves of high and low risk samples. **(L)** G3+G4 KM curves of high and low risk samples. **(M)** KM curves of drinking samples. **(N)** KM curves of non-drinking samples. **(O)** KM curves of HPV negative samples. **(P)** KM curves of HPV positive samples. **(Q)** Tabacco1 KM curves of high and low risk samples. **(R)** Tabacco2+3+4 KM curves of high and low risk samples.

TABLE 3 | Univariate and multivariate COX regression analyses of clinical factors.

Variables	Univariable analysis				Multivariable analysis			
	HR	95% CI of HR		*P*	HR	95% CI of HR		*P*
		Lower	Upper			Lower	Upper	
Age	1.017	1.005	1.030	0.007	1.022	1.008	1.035	0.001
Alcohol	1.025	0.792	1.326	0.850	0.927	0.710	1.212	0.581
T	1.099	0.962	1.256	0.164	0.907	0.776	1.059	0.216
N	**1.205**	1.045	1.389	**0.010**	**1.195**	1.015	1.406	**0.032**
Grade	1.096	0.915	1.313	0.318	1.051	0.867	1.274	0.612
Stage	**1.345**	1.138	1.589	**5.0E-04**	**1.310**	1.056	1.625	**0.014**
RiskScore	**1.913**	1.642	2.228	**2.0E-16**	**1.872**	1.613	2.173	**2.0E-16**

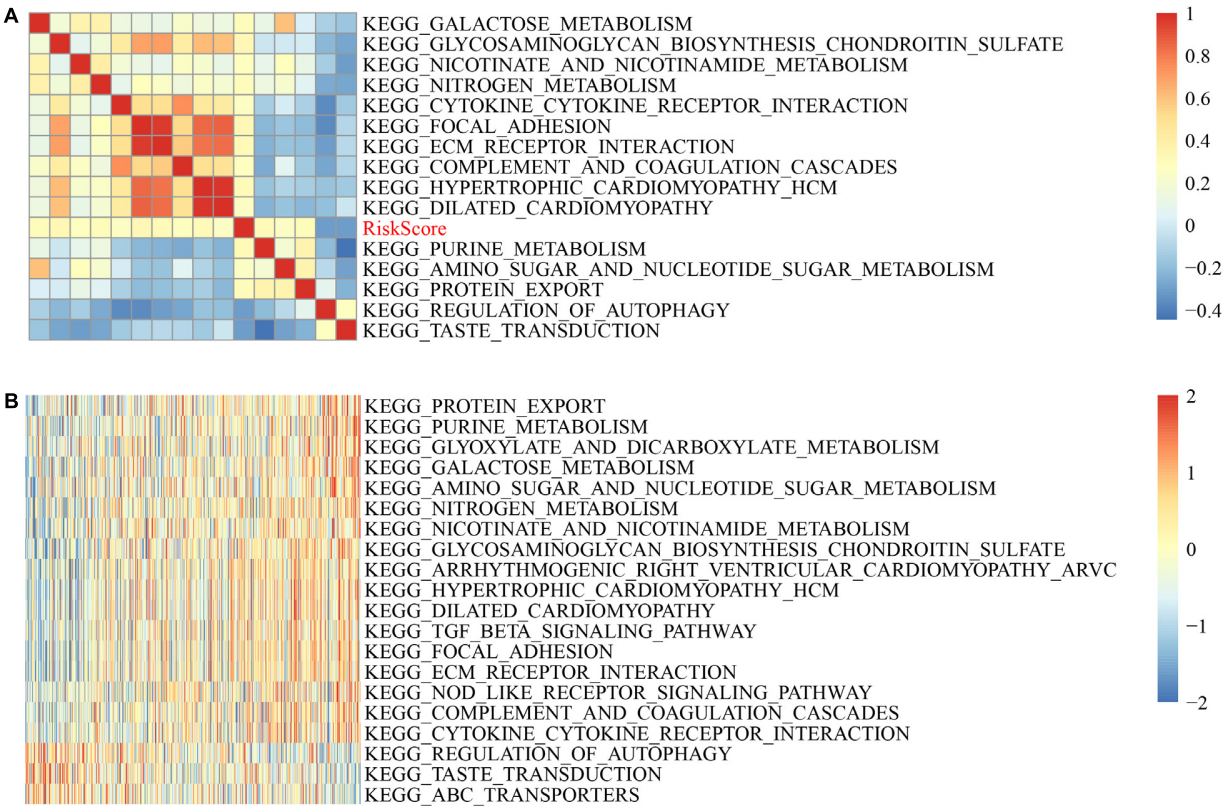

FIGURE 7 | GSVA-derived clustering heatmaps of different pathways. **(A)** Clustering of correlation coefficients between KEGG pathways and RiskScore with a correlation greater than 0.25 with risk scores. **(B)** The correlation between the KEGG pathway and the risk score is greater than 0.25, and the ssGSEA score in each sample changes with the increase in risk score. The horizontal axis represents the sample, and the risk score increases in turn from left to right.

HNSCC (Tang et al., 2013). These databases suggested that the current understanding of the relationship between HPV status and CSCs is still weak. It will be interesting to perform additional research for the underlying mechanism.

By applying WGCNA, an important system in bioinformatics used to generate gene co-expression networks to detect gene modules and identify key genes (Langfelder and Horvath, 2008; Li et al., 2018), gene modules that were correlated with mRNAsi indices based on the gene expression profile of HNSCC samples were initially identified. In these modules, blue one had the most considerable positive correlation with mRNAsi indices, while yellow one had the opposite. Functional annotation could be beneficial in evaluating the impact of these gene modules on HNSCC. Regarding the blue module, major biological processes were involved in regulating the mitotic phase, organelle fission and negative regulation of the cell cycle. KEGG enrichment pathways in the blue module encompassed DNA replication, p53 signaling pathway and the cell cycle. KEGG enrichment pathways in the yellow module were mainly involved in ECM–receptor interactions, PI3K-Akt signaling pathways, and MAPK signaling

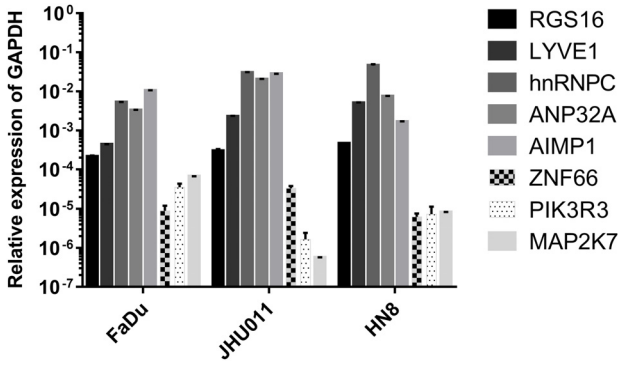

FIGURE 8 | The transcriptional expression level of eight mRNAsi in HNSCC cell lines.

pathways. These signaling pathways have been demonstrated to facilitate cell survival, self-renewal and apoptosis inhibition in many CSCs (Huang et al., 2017; Chen et al., 2020; Liao et al., 2020; Qin et al., 2020).

Key genes selected from mRNAsi correlated modules are currently employed in practice. Pan et al. (2019) screened 13 key genes based on mRNAsi associated gene modules in bladder cancer, which was shown to be related to stem cells. Pei et al. (2020) selected 12 mRNAsi based genes to be correlated with the survival of breast cancer patients. Zhang et al. (2020) showed 13 genes enriched in the cell cycle, which were increased due to the pathological stages of lung adenocarcinoma. These studies signified that there are inextricable links between key gene expressions and OS of patients. However, substantial evidence demonstrating that key genes may have predictive features in the clinical characteristics of cancer patients not yet elucidated. In the present study, 8-mRNAsi based signatures were established in predicting HNSCC. The riskscore was generated in samples of HNSCC based on expression patterns of these eight genes, which can serve as an independent predictor for OS in HNSCC patients (**Table 2**). The 8-mRNAsi based signature may also easily divide the HNSCC samples into high risk and low risk groups according to their various clinical characteristics required in the prognostic model for its potential use in clinical practice. Similar to our work, Cao and collaborators have evaluated the correlation between a three lncRNA signature patients OS with HNSCC by a log-rank test and univariable Cox regression. By OPLS-DA analysis and fold change selection, the three lncRNA signatures that can categorize patients into high and low risk groups have the highest predictive capacity. Comparatively, the same point is that univariable and multivariable Cox regression analysis were used to select the related genes in both studies. Otherwise, WGCNA and Lasso were performed in our study as the methods of dimensionality reduction for analyzing and selecting CSCs associated mRNA in HNSCC patients.

The 8-mRNAsi based prognostic model in our signatures includes *RGS16, LYVE1, hnRNPC, ANP32A, AIMP1, ZNF66, PIK3R3*, and *MAP2K7,* in which several genes have been reported to be linked with stemness features or be involved in cancer progression. *LYVE1*, lymphatic vessel endothelial hyaluronan receptor-1, has been identified as a biomarker of yolk sac endothelium and definitive hematopoietic stem and progenitor cells (HSPCs) by *Lyve1-Cre* labeling, where most HSPCs and erythro-myeloid progenitors were *Lyve1-Cre* lineage traced (Lee et al., 2016). *LYVE1* was thought to contribute to lymphangiogenesis in malignant tumors (Jackson et al., 2001). In the development of human embryonic stem cells, heterogeneous nuclear ribonucleoproteins (hnRNPs) has been identified as a critical regulator of physiologically relevant alternative cleavage and polyadenylation (APA) events that contribute to carcinogenesis by modulating the expression of genes that regulate cell proliferation and metastasis (Fischl et al., 2019). Silencing of hnRNPC can inhibit migratory and invasive activities by promoting miRNA-21 in brain tumor cells. Increased *hnRNPC* has been shown to contribute to cancer stemness and invasive potential in cancers (Park et al., 2012; Kleemann et al., 2018; Wu et al., 2018). However, the exact molecular function of *hnRNPC* needs to be explored in cancer stemness. *ANP32A*, acidic leucine-rich nuclear phosphoprotein-32A, expressed in normal tissue as well as multiple malignant tumors, several recent studies have indicated that *ANP32A* plays a significant role in cell proliferation, signal transduction, and other biological processes. Overexpression of *ANP32A* was associated with lymph node metastasis, which predicted poor survival in oral squamous cell carcinoma (OSCC) patients. Mechanical study indicates that *ANP32A* promotes tumor cell growth and may involve the inactivation of p38 and phosphorylation of Akt (Yan et al., 2017). *AIMP1* was identified as a cytokine that secretes in response to hypoxia and cytokine stimulation for involving cell proliferation regulation. A series of studies have shown that *AIMP1* can enhance wound healing by the mediation of fibroblast proliferation via ERK, and N-terminal domain (amino acids 6–46) of *AIMP1* was responsible for the stimulation of fibroblast proliferation (Park et al., 2005; Han et al., 2006). *AIMP1* peptide increased the expression of cyclin D1 and c-myc by stabilizing β-catenin through FGF receptor 2 (FGFR2)-mediated activation of Akt, which promotes the proliferation of bone marrow-derived mesenchymal stem cells (Kim et al., 2013). *ZNF66* is a member of the zinc finger transcription factor family which encounters many members and the gene coding for this protein is located on chromosome 19 in a fragile site region. Low mRNA expression of *ZNF66* is shown in head and neck cancers according to the TCGA dataset.[1] However, the correlations between the features of CSCs and *ZNF66* is still unclear, and additional studies need to be performed to explore the role of *ZNF66* in the stemness of HNSCC. *PIK3R3* is one of the regulatory subunits of *PI3K* that positively correlates with cell proliferation signatures (Phillips et al., 2006). Furthermore, the expression of *PIK3R3* increased in neoplastic tissues compared to non neoplastic in patients with gastric cancer (Zhou et al., 2012). However, higher expression of *PIK3R3* has been reported in cancer patients with satisfactory colorectal cancer outcomes as it facilitated the apoptosis of cancer cells (Ibrahim et al., 2018). *MAP2K7* is a mitogen-activated protein kinase, encodes *MMK7* and acts through the *JNK* pathway for cell cycle arrest and suppression of epithelial cancers (Schramek et al., 2011).

[1] https://www.proteinatlas.org

The robustness of the 8-mRNAsi based signature was validated across the TCGA test set and an external data set (GSE41613). Although these findings have been validated in HNSCC cell lines, further validation is still required in matched tissues of HNSCC patients. Additionally, the molecular process and signaling pathway obtained across the TCGA cases alone are inadequate and need to be confirmed through further investigation.

CONCLUSION

In our eight mRNAsi based signature, high expression of *RGS16, LYVE1, hnRNPC, ANP32A*, and *AIMP1* are correlated with a high risk of death as these genes focus in promoting cell proliferation and tumor progression, similar to stem cells. Regarding the other three genes, higher expression levels of *ZNF66, PIK3R3,* and *MAP2K7* are associated with a low risk of death. Interestingly, the molecular functions of these genes mainly concentrate on repressing the cell cycle and fostering apoptosis. Moreover, the present GSEA analysis discovered the mechanism regarding the KEGG pathway, which underlies the riskscore of the 8-mRNAsi based signature. Accordingly, to the best of our knowledge, all genes in the proposed mRNAsi based prognostic model have not been studied in HNSCC and may offer insight into the development of targeted therapies for HNSCC.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YT designed the study and analyzed the data. JW and YT carried out the manuscript. YT and CQ prepared the figures and tables. GZ, XC, ZC, YQ, MW, and ZL co-contributed to revising and polishing the manuscript. GC, XZ, and YL collated the data and supervised the study. All authors have read and approved the final submitted 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/fgene.2020.566159/full#supplementary-material

Supplementary Figure 1 | GO and KEGG pathway enrichment analysis in the blue module.

Supplementary Figure 2 | GO and KEGG pathway enrichment analysis in the yellow module.

Supplementary Figure 3 | Performance of the 8-mRNAsi based signature modelwith TCGA test dataset.

Supplementary Figure 4 | Performance of the 8-mRNAsi based signature modelwithGSE41613 database.

Supplementary Table 1 | The sequence of primers used for PCR in this study.

Supplementary Table 2 | 1518 genes information from yellow and blue modules.

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