ROLE OF RNA IN MOLECULAR DIAGNOSTICS OF CANCER

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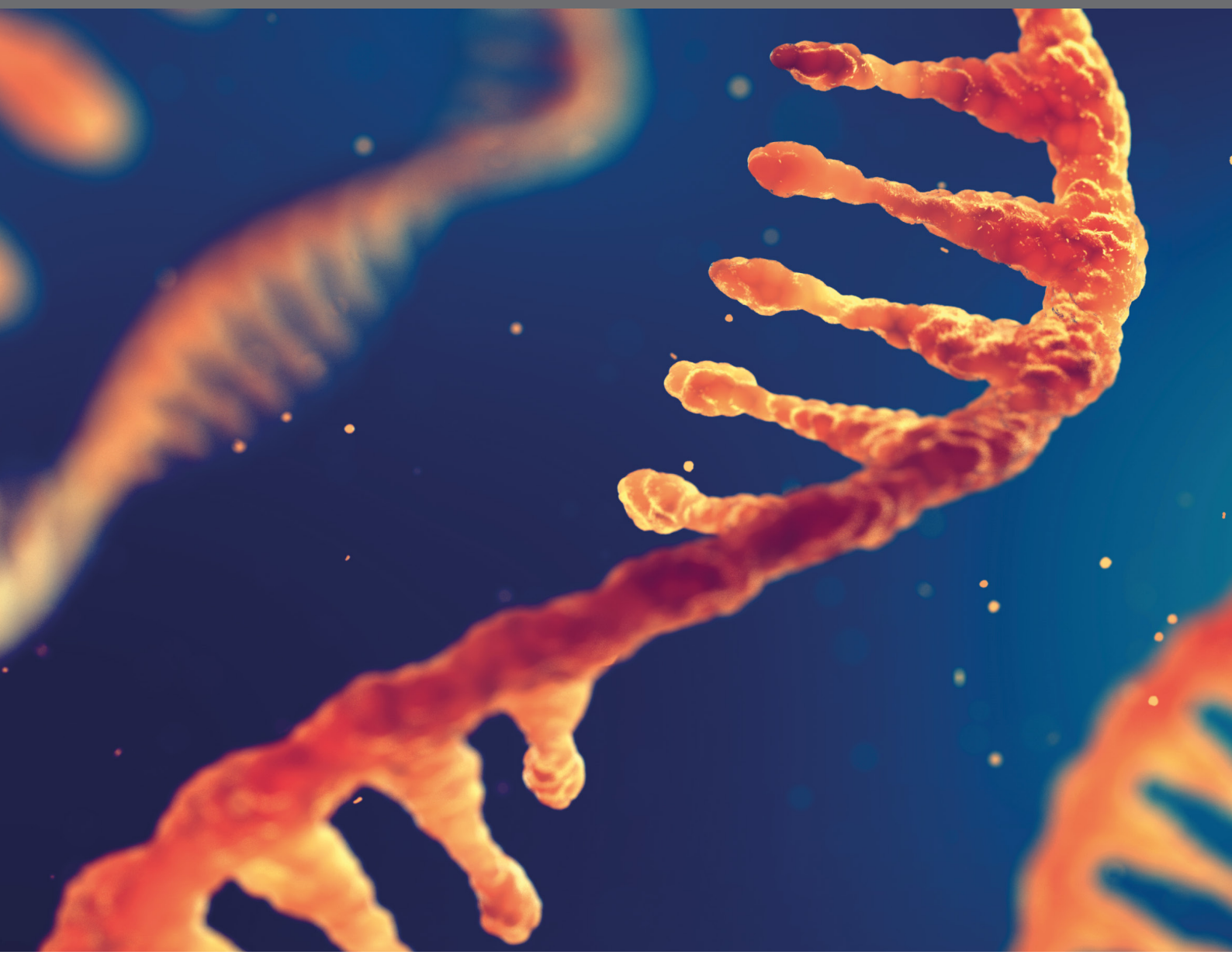

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ROLE OF RNA IN MOLECULAR DIAGNOSTICS OF CANCER

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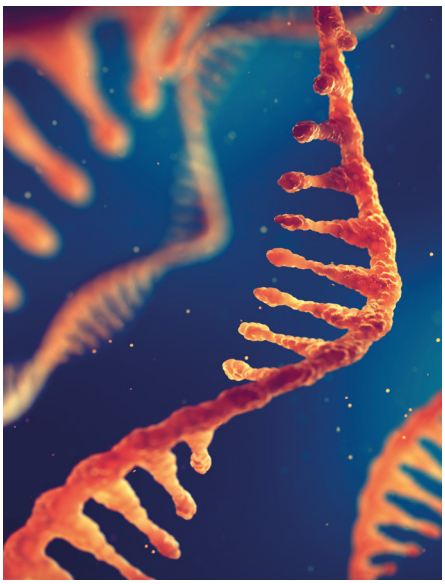

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EDITORIAL
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Editorial: Role of RNA in Molecular Diagnostics of Cancer

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Keywords: cancer, circulating cell-free RNA, liquid biopsy, molecular diagnostics, RNA

Editorial on the Research Topic

Role of RNA in Molecular Diagnostics of Cancer

The central dogma of molecular biology is an explanation of the flow of genetic information within a biological system. RNA is a dynamic bridge between the blueprint of life, DNA and the executor, protein. There is a broad spectrum of RNA, playing key roles in the living organisms, from physiological to pathophysiological processes.

RNA is essential for various biological roles, including coding, decoding, regulation, and expression of genes. In the world of RNA, about 2% is coding RNAs, while non-coding RNAs (ncRNAs) make up the majority (98%) of the transcriptomes. ncRNAs are RNA molecules transcribed from DNA but not translated into proteins. They regulate gene expression at the transcriptional and post-transcriptional levels. There are many types of ncRNAs, including tRNAs, rRNAs, long ncRNAs, and small RNAs (such as microRNAs, siRNAs, piRNAs, circRNA, snoRNAs, snRNAs, exRNAs). These molecules have fundamental roles in cells and many are stable in body fluids as extracellular RNAs. This Research Topic (RT) opens a platform for the update of the scientific development of RNA in molecular diagnostics of cancer.

MicroRNAs (miRNAs) are a class of ncRNAs that can be involved in the regulation of gene expression in cancer. Tian et al. found that miR-486-5p was significantly downregulated in non-small cell lung cancer (NSCLC) samples. PIK3R1 gene was confirmed to be the target of miR-486-5p. Overexpressed miR-486-5p effectively inhibited cell proliferation, invasion and successfully induced apoptosis *in vitro*. PIK3R1 also involved in the suppression of miR-486-5p on cell growth. They concluded that miR-486-5p might act as a tumor suppressor contributing to the progression of NSCLC, and miR-486-5p might be a diagnostic/prognostic biomarker and a potential therapeutic target for lung cancer. On the other hand, Zhang et al. identified the association of miR-153 with bladder cancer (BCA) prognosis using The Cancer Genome Atlas (TCGA) dataset. They found that miR-153 expression was downregulated in BCA tissues and cell lines. Reduced miR-153 expression was associated with poor prognosis of advanced stage patients. Furthermore, miR-153 expression inhibited BCA cell growth by promoting tumor cell apoptosis, migration, invasion, and epithelial–mesenchymal transition (EMT) *in vitro* and tumor xenograft growth *in vivo*, it could also suppress human umbilical vein endothelial cell and chicken chorioallantoic membrane angiogenesis. In addition, they found that miR-153 targeted IDO1 expression and inhibited BCA cell tryptophan metabolism through inhibiting IL6/STAT3/VEGF signaling. They thus concluded that miR-153 exerted anti-tumor activity in BCA by targeting IDO1 expression, miR-153 might serve as a novel therapeutic target for BCA patients.

Emerging evidence is accumulating that tumor cells release substantial amounts of RNA into the bloodstream, which strongly resist RNases in the blood. This liquid biopsy contains circulating RNAs which are usually packed in extracellular vesicles and have considerable potential as minimally-invasive biomarkers, since they are stable and some have been associated with disease

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phenotypes (Cheung et al., 2019). miRNAs are endogenous non-coding small RNA molecules that can be secreted into the circulation and exist in remarkably stable forms. Numerous efforts have been devoted to identifying suitable extracellular miRNAs for non-invasive biomarkers in cancer. However, not only technical challenges, but also cognitive challenges need to be overcome before practical application to be achieved. Cui et al. reviewed circulating miRNAs in terms of their biological function and basic transport carriers; extracellular cell communication process; roles as reliable cancer biomarkers and used in targeted cancer therapy; and challenges for clinical application.

Apart from blood, saliva is an easily accessible liquid biopsy. Based on gene set enrichment analysis, Yu et al. found that programmed death-ligand 1 (PD-L1) expression positively correlated with inflammation in periodontitis. They found that the levels of PD-L1 mRNA in salivary exosomes were higher in periodontitis patients than in controls. They claimed that this is the first protocol on isolating and detecting exosomal RNA from saliva of periodontitis patients. Their assay of exosomes-based PD-L1 mRNA in saliva has potential to distinguish periodontitis from the healthy, and the levels correlate with the severity/stage of periodontitis.

Recently, long non-coding RNAs (lncRNAs) have been reported to be highly implicated in the development and progression of breast cancer. Wu et al. reviewed that the majority of lncRNAs exert their roles through the regulation of invasion, migration, EMT, and metastasis process. Not only tumor progression, increasing evidence showed that lncRNAs play a role in chemotherapy resistance. A number of publications has reported the link between miRNA and lncRNAs. In this Research Topic, Li et al. demonstrated that lncRNA DLEU1 was upregulated in BCA tissues and high DLEU1 expression correlated with a poorer prognosis of BCA patients. The authors demonstrated that DLEU1 induced cell proliferation, invasion, and cisplatin resistance of BCA cells by de-repressing the expression of HS3ST3B1 through sponging miR-99b. Low miR-99b and high HS3ST3B1 levels were correlated with worse prognosis in patients with BCA. Ectopic expression of HS3ST3B1 or inhibition of miR-99b reversed DLEU1 knockdown mediated suppression of cell proliferation, invasion, and cisplatin resistance. They concluded a novel role for the DLEU1/miR-99b/HS3ST3B1 axis in regulating proliferation, invasion, and cisplatin resistance of BCA cells.

Recently, lncRNA has been shown as a competing endogenous RNA (ceRNA) to hinder miRNA functions that participate in posttranscriptional regulatory networks in tumors. However, few studies have focused on predicting cholangiocarcinoma (CCA) prognosis, and no systematic examination has been performed on the prognostic value of ceRNA in CCA. Long et al. performed a comprehensive comparison on RNA-sequencing and miRNA profiles from TCGA database. They further validated the mRNA expression levels in Gene Expression Omnibus. In the survival analysis, they found that 26 lncRNAs, 3 miRNAs, and 13 mRNAs were prognostic biomarkers for patients with CCA. They disclosed a better understanding of the ceRNA network involved in CCA biology that may improve CCA diagnosis and prognosis.

On the other hand, circular RNA (circRNA) is a novel class of lncRNA, resulting from pre-mRNA back splicing with a stable circular conformation that regulates various biological processes. The aberrant expression of circRNA and its impact on a number of cancers is still unclear. Nevertheless, Jamal et al. reviewed the updated functional roles of circRNAs in acute myeloid leukemia (AML). They also provided the mechanistic insights, discussed the challenges and opportunities associated with circRNA-based diagnostic and therapeutic development in AML.

Apart from circulating cell-free DNA which has been extensively studied, the detection and quantification of circulating cell-free RNA has gained great attention. Owing to the development of high-throughput transcriptomic analyses, numerous RNA splice variants and circRNAs have been found to be differentially expressed in tumor patients compared to controls. de Fraipont et al. reviewed the contribution of these RNA splice variants and circRNAs to tumor progression, dissemination, or drug response in preclinical models. The potential of circRNAs and mRNA splice variants as candidate biomarkers for the prognosis and the therapeutic response of NSCLC in liquid biopsies was well-discussed. We know that early detection is crucial for patient management, Prof Wong led a study which making use of custom designed colorectal cancer-associated targeted RNA-Seq panel on the detection of plasma mRNA in colorectal adenoma patients and normal healthy subjects. They found that GSK3A and RHOA in adenoma patients were significantly lower than those in normal healthy subjects (Xue et al., 2019).

Among the ncRNA, there is a class called small nucleolar RNAs (snoRNAs) which can be divided into C/D box and H/ACA box snoRNAs. Emerging evidence has demonstrated that the mutations and aberrant expression of snoRNAs involved in cell transformation, tumorigenesis, and metastasis. Actually snoRNAs may serve as biomarkers and potential therapeutic targets of cancer. Liang et al. reviewed the biogenesis and functions of snoRNAs, as well as the association of snoRNAs in different types of cancers and their potential roles in cancer diagnosis and therapy.

Some landmark translational researches help cancer patients in a remarkable manner, such as MammaPrint, Oncotype DX, etc. (Matikas et al., 2019). It is appealing to develop a multi-gene panel to predict the treatment response or patient prognosis. For example, there is a six-hypermethylated gene panel associated with poor survival in patients with nasopharyngeal carcinoma (NPC), demonstrating its potential usefulness as a prognostic biomarker to clinicians in NPC management (Jiang et al., 2015). In this RT, Liu et al. constructed a co-expression network to identify hub genes related to the International Staging System (ISS) stage of multiple myeloma (MM). Further analyses including functional analysis of hub genes, univariate Cox proportional hazard regression analysis as well as least absolute shrinkage and selection operator penalized Cox proportional hazards regression model were employed. The hub genes were further validated in the test set and an independent validation cohort. Finally, nine hub genes (HLA-DPB1, TOP2A, FABP5, CYP1B1, IGHM, FANCI, LYZ, HMGN5, and BEND6) were

identified to build a 9-gene prognostic signature. They found that the relapsed MM and stage III MM was associated with a high risk score based on the signature. Further prospective clinical trials are warranted to test its clinical utility.

This RT will be published as an Ebook. We wish that all these efforts may enhance our understanding of the RNA world and provide some insight on its potential clinical utilization. RNA is diverse, dynamic, and does not work alone, future research directions may involve multifunctional biomolecular diagnostic markers and technology platforms. We envision a more comprehensive understanding of cancer biology by multi-omics approaches which represent distinct aspects of the central dogma of molecular biology (Cho, 2010; Paczkowska et al., 2020).

AUTHOR CONTRIBUTIONS

WC drafted the manuscript. LC and CW edited the manuscript. All authors approved the manuscript.

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ORIGINAL RESEARCH
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Construction and Investigation of a lncRNA-Associated ceRNA Regulatory Network in Cholangiocarcinoma

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Background/Aims: As a type of malignant tumor commonly found in the bile duct, cholangiocarcinoma (CCA) has a poor prognosis. Long non-coding RNA (lncRNA) has recently drawn increasing attention because it functions as a competing endogenous RNA (ceRNA) to hinder miRNA functions that participate in posttranscriptional regulatory networks in tumors. Therefore, to investigate the mechanisms of CCA carcinogenesis and to enhance treatment efficiency, the expression profiles, including lncRNA, miRNA, and mRNA data, were comprehensively integrated and analyzed in this study.

Methods: A comprehensive comparison was performed on the RNA-sequencing and miRNA profiles data of 36 CCA samples and 9 normal samples from The Cancer Genome Atlas (TCGA) database. Then, a dysregulated lncRNA-related ceRNA network was established by using four public databases.

Results: In summary, 1,410 lncRNAs, 64 miRNAs, and 3,494 mRNAs appeared as genes that were aberrantly expressed in CCA. Then, a dysregulated ceRNA network related to the lncRNAs was constructed. The network included 116 lncRNAs, 13 miRNAs and 60 mRNAs specific to CCA. The survival analysis showed that, among them, 26 lncRNAs, 3 miRNAs, and 13 mRNAs were prognostic biomarkers for patients with CCA. Finally, three mRNAs were selected for validation of their expression levels in the Gene Expression Omnibus (GEO) database. The results indicated that the expression of those genes was highly consistent between the TCGA and GEO databases.

Conclusions: The findings in this study provide a better understanding of the ceRNA network involved in CCA biology and lay a solid foundation for improving CCA diagnosis and prognosis.

Keywords: competing endogenous RNA network, long non-coding RNA, cholangiocarcinoma, prognosis, microRNA

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignancy most commonly observed in the bile duct and the second most common primary hepatocellular carcinoma (HCC) (1). CAA can be divided into three subtypes, namely, intrahepatic distal (dCCA), perihilar (pCCA), and intrahepatic (iCCA) cholangiocarcinoma. Although such malignancy usually occurs in Asia, an obvious increase in incidence has recently been observed in North America and Europe (2). In addition, CCA has a dismal prognosis (3). Curative treatment for patients with CCA may depend on surgery; however, most patients are diagnosed at an advanced stage of disease, and recurrence can occur frequently after resection (4). Patients with an advanced tumor stage have a dismal 5-year survival rate of below 5% (4). Given the lack of effective treatment options at an advanced stage of disease, the early detection of CCA is essential for providing patients a curative therapeutic approach and to maximize clinical outcomes (4). Therefore, prognostic biomarkers might help guide treatment decisions with respect to patient life expectancy and develop personalized treatments for individual CCA patients.

Long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides, act as non-protein-coding transcripts (5). Because they do not encode proteins, they were originally thought to be "junk DNA." However, increasing attention has been recently paid to the application of lncRNAs in the biology of cancers because lncRNAs can participate in carcinogenesis and cancer metastasis, as revealed by several studies (6). Nevertheless, it is unclear whether lncRNAs regulate gene expression. The regulatory function of lncRNAs on gene expression at the transcription, post-transcription and translation levels has been reported in the form of both theoretical and experimental results, but the complete influencing mechanism of lncRNAs on cancer has not been elucidated (6).

Salmena et al. put forth a hypothesis about competing endogenous RNA (ceRNA) in 2011, revealing that lncRNA, mRNA, and other types of RNAs can hinder miRNA function by virtue of shared microRNA response elements (MREs) as natural miRNA sponges (7). Many studies have proven this hypothesis via experiments, showing that lncRNA can compete for miRNA as a type of ceRNA to communicate with mRNA (8). Due to similar sequences with targeted miRNA, lncRNA is able to separate miRNA from mRNA. For example, under the upregulation of oxidative stress, H19 and HULC can target IL-6 and CXCR4 by virtue of ceRNA patterns of sponging let-7a/let-7b and miR-372/miR-373, respectively, to moderate the invasion and migration of CCA cells (9). The lncRNA TUG1 can competitively bind itself to miR-335-5p to regulate ROCK1, followed by inducing the migration and invasion of osteosarcoma cells (10). It is believed that exploring RNA interactions is of vital significance for the treatment of cancer. Specific lncRNA-miRNA-mRNA ceRNA networks have been constructed for esophageal squamous cell carcinoma, cutaneous melanoma and human lung squamous cell carcinoma on the basis of the abovementioned theory (11–13). Nevertheless, there remain insufficient comprehensive analyses on the lncRNAs and miRNAs related to CCA at the genome-wide scale, particularly on the basis of high-through detection.

The Cancer Genome Atlas (TCGA) database, which is available to the public, provides abundant resources for biological discovery and data mining because it collects data from approximately 10,000 patients and clinicopathological information related to over 30 types of human cancers (14, 15). Recently, the study with TCGA in CCA was published and this integrated analysis of DNA methylation, copy number, RNA expression, and somatic mutations led to a molecular classification and identified an IDH mutant-enriched subtype with distinct molecular characteristics (14). In the present study, we extracted expression data from TCGA including data from 36 tumor samples and 9 adjacent non-tumor samples (14). Here, we used differentially expressed genes (DEGs) between CCA and the adjacent liver to construct a ceRNA network. In total, 1,410 lncRNAs, 64 miRNAs, and 3,494 mRNAs were identified to be aberrantly expressed in CCA and were used to construct the aberrant ceRNA network related to the lncRNAs. The ceRNA network consisted of 116 lncRNAs, 13 miRNAs, and 60 mRNAs. Survival analyses showed that, among them, 26 lncRNAs, 3 miRNAs, and 13 mRNAs were prognostic biomarkers for patients with CCA. In conclusion, novel lncRNAs, miRNAs, and mRNAs were identified as potential prognostic biomarkers and therapeutic targets specific to CCA. More importantly, the proposed ceRNA network may allow a better understanding of the regulatory mechanism of ceRNA mediated by lncRNA in the pathogenesis of CCA.

MATERIALS AND METHODS

Data Collection and Pre-processing

The RNA expression profiles (level 3) and the clinical information of patients with CCA were downloaded from TCGA. Specifically, the study included all samples with expression data ($n = 45$) of patients with CCA in the TCGA database, including 36 CCA tumor samples and 9 non-tumor samples. LncRNAs and mRNAs were quantified on the basis of the Genome Research Project of ENCyclopedia of DNA Elements (GENCODE) (GRCh38) catalog (http://www.gencodegenes.org/). The transformed data (sense_overlapping, lincRNA, 3prime_overlapping_ncrna, processed_transcript, antisense and sense_intronic) were considered lncRNAs. In summary, 19,660 mRNAs and 1,4254 lncRNAs derived from RNA sequencing (RNA-Seq) as well as 1,881 miRNAs derived from miRNA-Seq were retrieved. The sequencing data were derived using the Illumina HiSeq RNA-Seq and Illumina HiSeq miRNA-Seq platforms. This study was carried out strictly following TCGA publication guidelines. As the data came from TCGA, there was no need for ethics committee approval. Another CCA patient cohort that included 163 samples (104 CCA samples and 59 surrounding liver samples) was downloaded from the GEO database under accession number GSE26566 and was applied to examine the expression of mRNA biomarkers as the validation cohort (16). In addition, the gene expression profiles from GSE89749 (including 118 CCA samples) and GSE76297 (including 91 CCA samples) also were obtained from

the GEO database to validate the relationships between lncRNAs and mRNAs.

Identification of Significantly DEGs

Differential expression analyses were performed for the identification of differentially expressed lncRNAs, mRNAs and miRNAs (hereafter referred to as "DElncRNAs," "DEmRNAs" and "DEmiRNAs," respectively) between the non-cancerous samples and the CCA samples using the "edgeR" packages in R (17). DElncRNAs, DEmiRNAs, and DEmRNAs were selected based on the same cut-off value corresponding to the false discovery rate (FDR) adjusted by the Benjamini-Hochberg method. FDR <0.05 and |log fold change (FC)| >1 were taken as the cut-off criteria. Heatmaps were generated by hierarchical clustering analysis and utilized to identify the gene expression differences between the normal samples and the CCA samples.

Construction of the ceRNA Network

The miRcode database was used to predict which DElncRNAs and DEmiRNAs could interact with each other (18). DEmRNAs targeted by DEmiRNAs were retrieved based on the TargetScan, miRDB, and miRTarBase databases, and only those recognized by these three mentioned databases served as candidate DEmRNAs (19–21). The next step was to establish the lncRNA-miRNA-mRNA ceRNA network on the foundation of the interactions between DElncRNAs and DEmiRNAs and between DEmiRNAs and DEmRNAs. The established network was visualized by "ggalluvial" R package.

Functional and Pathway Enrichment Analyses

To clarify the potential biological process of DEmRNAs associated with the ceRNA network, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) online tool (https://david.ncifcrf.gov/) to analyze the Gene Ontology (GO) functional enrichment of DEmRNAs (22). Default parameters were set for the analysis. The whole human genome was set as the background, and functional categories with $P < 0.05$ were considered significant. To understand the potential pathways of the DEmRNAs participating in the ceRNA network, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEmRNAs was performed using the KO-Based Annotation System (KOBAS) online tool (http://kobas.cbi.pku.edu.cn/index.php) (23). KEGG pathways were also evaluated for significance ($P < 0.05$).

Construction of Protein-Protein Interaction (PPI) Network

We adopted the Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/) database to build the PPT network of DEmRNAs, aiming to clarify the interaction among DEmRNAs (24). Additionally, Cytoscape software was used to embody the constructed PPI networks.

Survival Analysis

As the ceRNA network proposed a large number of potential DElncRNAs, DEmiRNAs, and DEmRNAs, the association among these genes and the prognosis of patients with CCA were estimated to unveil to what extent and in which aspects these genes play a role in the overall survival of patients with CCA. We used the survival package in R to perform the Kaplan–Meier survival analyses of the association between the expression of DElncRNAs, DEmiRNAs, and DEmRNAs in the ceRNA network and the prognosis of patients with CCA.

Clinical Feature Analysis of ceRNAs Network

We extracted clinical information from TCGA database. Clinical features such as vascular invasion, histologic grade and neoplasm histologic grade were chosen to analyze the correlation between these features and ceRNAs.

Validation of DEmRNAs

The GSE26566 dataset was adopted for validating the expression of candidate genes (25). The transcriptome profiles in the GSE26566 (Platform: GPL6104) dataset including 104 CCA samples and 59 non-tumor liver samples (16).

Functional Prediction of the DEmRNAs

To infer the potential functions of the DEmRNAs in the ceRNA network, gene set enrichment analysis (GSEA) of the DEmRNAs was performed. Thirty-five CCA samples in the TCGA database were classified into two groups (high vs. low) according to the median expression level of the DEmRNAs in the RNA sequencing data. The reference gene sets were the annotated gene sets of c2.cp.kegg.v6.1.symbols.gmt in the Molecular Signatures Database (MSigDB). The cut-off criteria was $P < 0.05$.

RNA Extraction and qPCR Validation

Eleven paired CCA and corresponding normal liver tissue samples were attained from patients with CCA in the Peking Union Medical College Hospital (PUMCH). All the diagnoses were according to pathological assessments. Upon removal of the surgical specimen, each sample was immediately frozen in liquid nitrogen and stored at −80°C prior to RNA isolation and qPCR analysis.

We extracted RNA from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using the HiScript III RT SuperMix for qPCR (+g DNA wiper) (Vazyme Biotech, NanJing, China). qPCR was performed with a AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, NanJing, China) via a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The sequence of PCR primers of SLC43A1 were GGACGTGGAAGCTCTGTCTC (Forward primer) and GCAGCGTGAGTGAAGTGAAC (Reverse primer). The PCR cycling conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 10 sec, 60°C for 34 s, dissociation at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The results were analyzed using the $2^{-\Delta\Delta CT}$ method. The qPCR reactions were all repeated three times. The paired *t*-test was used for normally distributed data; otherwise, the Wilcoxon rank test for paired data was used to evaluate whether a gene was differentially expressed between tumor samples and normal samples.

RESULTS

Identification of Differentially Expressed lncRNA, miRNA and mRNA

All samples with expression data ($n = 45$) of patients with CCA in the TCGA database, including 36 CCA tumor tissues and 9 adjacent non-cancerous tissues were enrolled in our study (**Table S1**). Differential expression analysis was performed based on comparisons of the expression of 14,254 lncRNAs, 19,660 miRNAs and 1,881 mRNAs between 36 CCA and 9 adjacent normal liver tissue samples in the TCGA database. Using thresholds of FDR <0.05 and |log FC| >1, 1,410 DElncRNAs (916 upregulated and 494 downregulated), 64 DEmiRNAs (34 upregulated and 30 downregulated), and 3,494 DEmRNAs (2,184 upregulated and 1,310 downregulated) between CCA and adjacent normal liver tissues were identified (**Figure 1A**; **Table S2**). Clustering analysis and principal component analysis of DElncRNAs, DEmiRNAs, and DEmRNAs suggested that CCA samples may be distinguished from normal samples based on the expression levels of DElncRNAs, DEmiRNAs, and DEmRNAs (**Figures 1B,C**).

Prediction of DelncRNA-DEmiRNA Interactions by miRcode

We used the miRcode database to predict the miRNA-targeted lncRNAs. The constructed ceRNA network only included the interactions between DEmiRNAs and DElncRNAs; thus, 328 interactions between 117 DElncRNAs (AL591845.1, KIAA0087, and H19, etc.) and 14 DEmiRNAs (hsa-mir-96, hsa-mir-182, and hsa-mir-122, etc.) were identified (**Table S3**).

Prediction of DEmiRNA-Targeted DEmRNAs

We mapped the abovementioned 14 DEmiRNAs into the TargetScan, miRDB, and miRTarBase databases and searched for the target DEmRNAs. Sixty DEmRNAs that interacted with 13 of the 14 DEmiRNAs in all 3 datasets were selected (**Table S4**). After removing the remaining 1 DEmiRNA and the corresponding lncRNAs, finally 116 DElncRNAs, 13 DEmiRNAs, and 60 DEmRNAs were obtained to build the ceRNA network (**Figure 2**; **Table S4**). In addition, we calculated the connection degree of each gene by topology to clarify its importance in the ceRNA network (**Figure 2**; **Table S6**). KIAA0087 (connection degree = 8), hsa-mir-211 (connection degree = 50), and KIAA1549 (connection degree = 2) were considered the most important genes among the lncRNAs, miRNAs, and mRNAs, respectively (**Figure 2**; **Table S6**). Because hsa-mir-211 was the gene with the highest connection degree in the ceRNA network (connection degree = 50), we concluded that it might exert a strong influence on CCA pathogenesis. The ceRNA network also shows that DElncRNAs interact with DEmRNAs in an indirect manner.

Functional Enrichment Analysis and Protein-Protein Interaction Network Construction

To determine the biological functions and pathways of the 60 DEmRNAs in the constructed ceRNA network, we used the DAVID database to analyze the GO functional enrichment and the KOBAS database to analyze KEGG pathway enrichment. This analysis revealed the enrichment of 15 GO terms and 7 KEGG pathways (with $P < 0.001$ and 0.0001, respectively; **Tables S7**, **S8**). The top GO terms were "sequence-specific DNA binding," "sensory organ development" and "growth factor activity" (**Figure 3A**; **Table S7**). According to KEGG pathway analyses, the most significant pathways identified were "endocrine resistance," "estrogen signaling pathway" and "TNF signaling pathway" (**Figure 3B**; **Table S8**). In addition, we constructed PPI networks to explore the relationship among DEGs via the STRING database, which was visualized by Cytoscape software (**Figure 3B**).

Association Between Key Genes and Overall Survival

We performed a log-rank test and Kaplan-Meier survival analysis for each DElncRNA, DEmiRNA, and DEmRNA in the constructed ceRNA network to obtain RNAs that exhibited a close relation to the prognosis of patients with CCA. Twenty-six DElncRNAs, 3 DEmiRNAs, and 13 DEmRNAs were identified as RNAs associated with overall survival based on their respective optimal cutoffs (**Figure 4**; **Tables S9–S11**). Among these, 17 DElncRNAs, 2 DEmiRNAs, and 7 DEmRNAs seemed to exhibit protective function, as the prognosis of patients with higher expression levels of these RNAs was longer than that in patients with lower expression levels. In contrast, the remaining 9 DElncRNAs, 1 DEmiRNA, and 6 DEmRNAs were regarded as risky, as there was a negative relationship between their expression and the prognosis of CCA patients.

Positive Correlation Among the Expression Levels of ceRNAs

According to ceRNA theory, lncRNAs can positively regulate mRNAs by competitively compete with miRNAs for the binding sites of miRNAs. To validate this finding, a regression analysis was carried out between DElncRNAs and DEmRNAs targeted by hsa-mir-211, which was the gene with the highest degree of connections in the ceRNA network (connection degree = 50). Strong positive correlations were found between DElncRNAs and DEmRNAs targeted by hsa-mir-211 (minimum requirement of an interaction score >0.4; **Table S12**). **Figure 5** showed the top three correlation coefficients of interactions, in which LINC00261 interacts with SLC43A1, HOTAIR interacts with HOXC8, and LINC00242 interacts with ELOVL6 under the regulation of hsa-mir-211.

Validation of DEmRNAs in the ceRNA Network

To verify our analysis, the expression levels of SLC43A1, HOXC8, and ELOVL6 were assessed in 104 CCA tumor

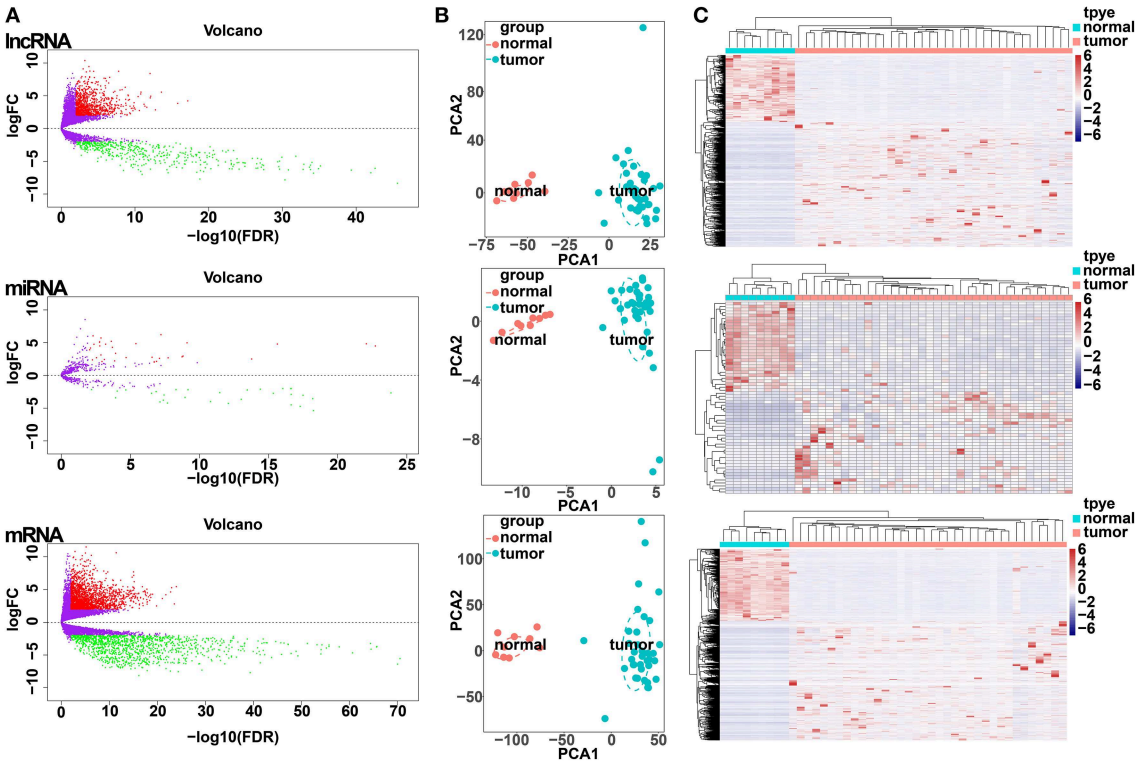

FIGURE 1 | Differentially expressed gene (DEG) analysis. **(A)** Volcano plots of differentially expressed lncRNAs (upper panel), miRNAs (middle panel), and mRNAs (lower panel). The Y-axis represents the negative log (base 10) of the FDR, and the X-axis represents the log (base 2) of FC. Each point represents a gene. The dots in green denote downregulated DEGs, the dots in red denote upregulated DEGs, and the dots in black denote non-DEGs. **(B)** Principal components analysis (PCA) plot of the differentially expressed lncRNAs (upper panel), miRNAs (middle panel), and mRNAs (lower panel). The tumor samples (green, $n = 36$) and normal samples (red, $n = 9$) are plotted along the axis about the first two principal components (PC1 and PC2). **(C)** Heatmap plots of the differentially expressed lncRNAs (upper panel), miRNAs (middle panel), and mRNAs (lower panel). The horizontal axis represents the samples, and the upper horizontal axis represents the sample clusters. The vertical axis represents the DEGs, and the left vertical axis shows the DEG clusters. Red represents upregulated genes, while green represents downregulated genes.

tissues and 59 adjacent non-tumor liver tissues. Consistent with our analysis findings, the mean expression levels of SLC43A1 and ELOVL6 were significantly lower while HOXC8 was significantly higher in CCA samples than in non-tumor liver samples in the GSE26566 dataset (**Figures 6A,B**). The results proved the reliability of our analysis.

GSEA of the DEmRNAs

To identify the biological pathways related to SLC43A1, HOXC8, and ELOVL6, which were highly expressed according to the TCGA database, we performed GSEA in the CCA tissues based on the TCGA database (**Figure 6C**). CCA tissues in the SLC43A1 high expression group were most significantly enriched for "Parkinson's disease" (**Table S13**); CCA tissues in the HOXC8 high expression group were most significantly enriched for "primary immunodeficiency" (**Table S14**); and CCA tissues in the ELOVL6 high expression group were most significantly enriched for "complement and coagulation cascades" (**Table S15**).

Validation of Positive Correlations Between lncRNAs and mRNAs

To make our results more reliable, we also verified the relationship between lncRNA and mRNA in other independent datasets (GSE89749 and GSE76297). Since among the correlation coefficients between DElncRNAs and DEmRNAs targeted by hsa-mir-211, the relationship between LINC00261 and SLC43A1 have highest correlation coefficient, we only verified the relationship between LINC00261 and SLC43A1. The results of the correlation analysis of these two datasets (GSE89749 and GSE76297) are consistent with the results of TCGA (**Figures 5**, **7A,B**). In addition, to check the credibility of the bioinformatics results, we validated the expression of SLC43A1 by using qPCR. Paired *t*-tests were used to analyze the differences in expression of the SLC43A1 between 11 CCA samples and 11 adjacent non-cancerous samples. The qPCR results from 11 CCA patients were consistent with the bioinformatics results (**Figures 6A,B**, **7C**).

Clinical Feature Analysis of ceRNAs

To further study the ceRNAs, the correlation between the ceRNAs involved in the ceRNA network and clinical features including

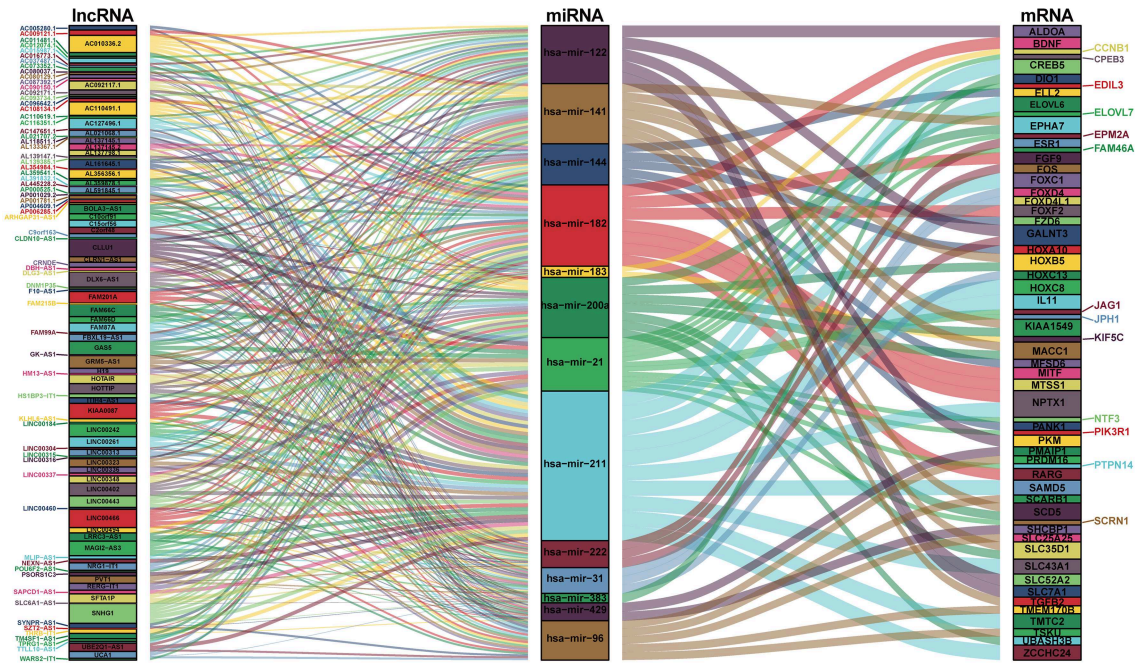

FIGURE 2 | Sankey diagram for the ceRNA network in cholangiocarcinoma. Each rectangle represents a gene, and the connection degree of each gene is displayed based on the size of the rectangle.

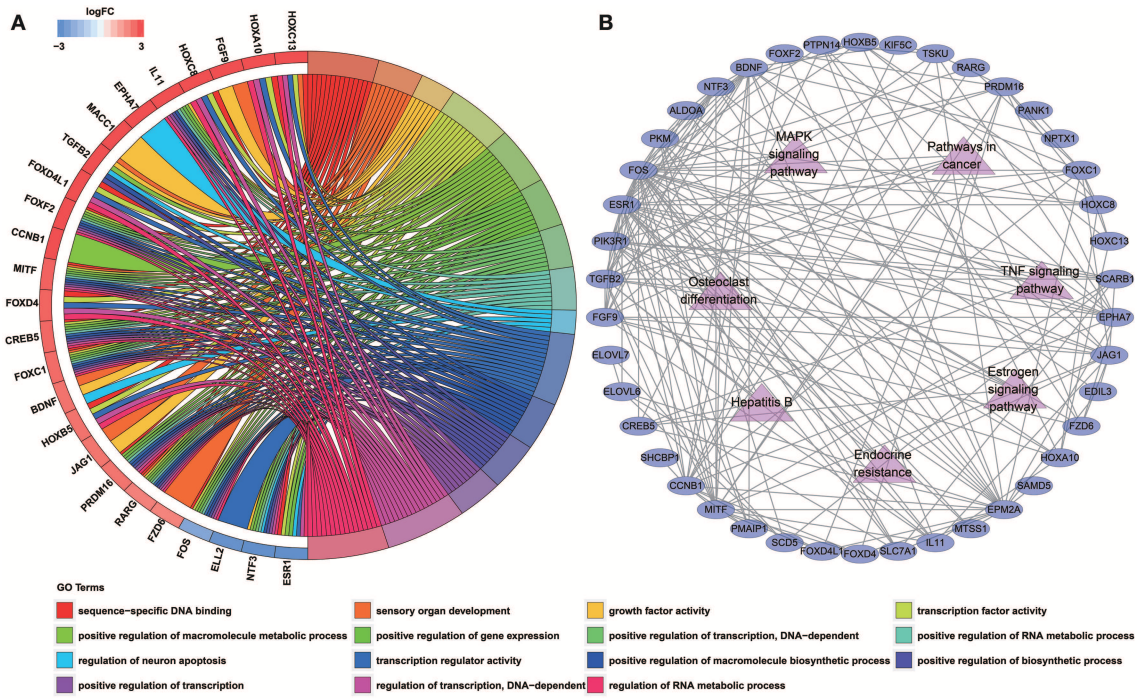

FIGURE 3 | Functional enrichment analysis of 60 DEmRNAs. **(A)** Chord diagram showing enriched GO clusters for the 60 DEmRNAs. In each chord diagram, genes contributing to their respective enrichment are shown on the left, and enriched GO clusters are shown on the right. Downregulated DEmRNAs are displayed in blue, whereas upregulated DEmRNAs are displayed in red. Each GO term is represented by one colored line. **(B)** PPI networks of the DEmRNAs. Ellipse represents DEmRNA, and edge represents the interaction between two proteins. Triangles represent pathways in which 60 DEmRNAs were enriched.

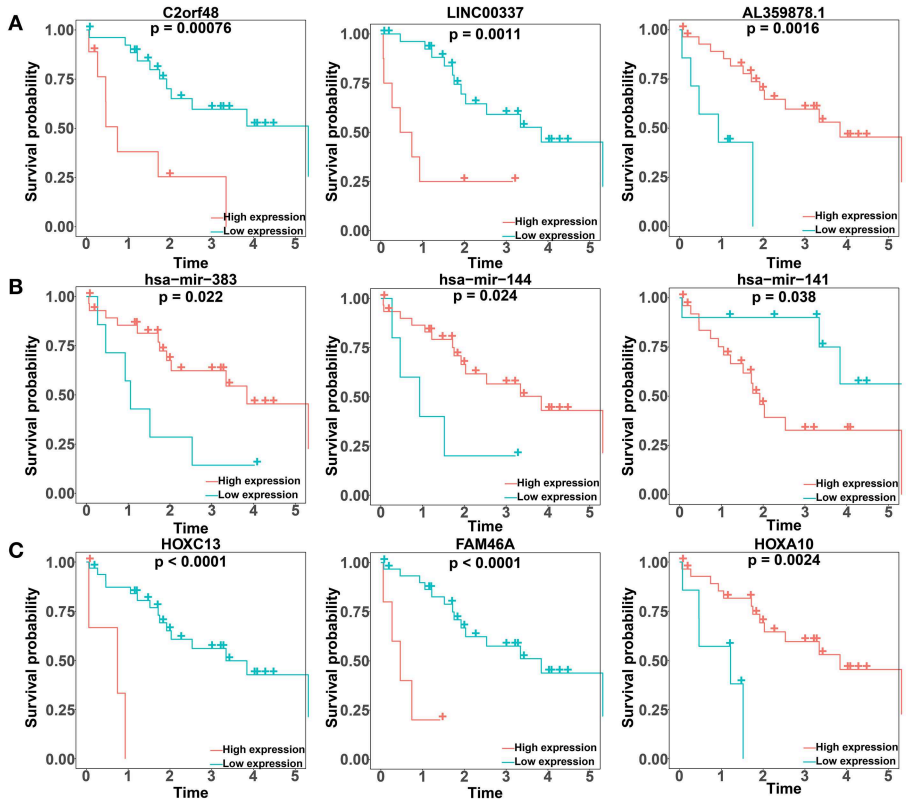

FIGURE 4 | Kaplan–Meier survival curves for the top three lncRNAs, miRNAs, and mRNAs related to overall survival based on their optimal cutoffs in cholangiocarcinoma. The horizontal axis represents the overall survival time in years; the vertical axis represents the survival probability.

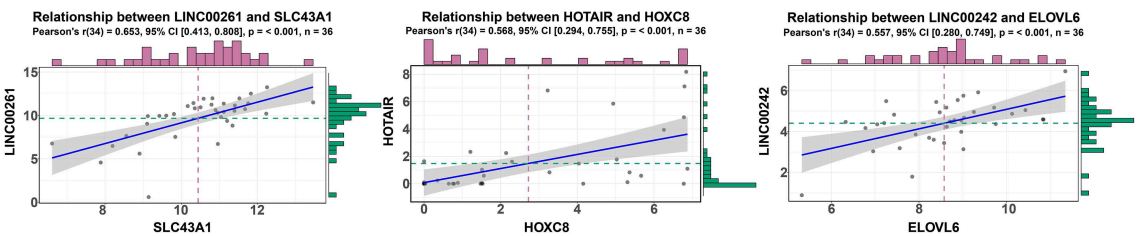

FIGURE 5 | Regression analysis between the expression levels of DElncRNAs and DEmRNAs targeted by hsa-miR-211 in the ceRNA network (showing the top three correlation coefficients). The horizontal axis denotes the expression of the DEmRNAs, and the vertical axis represents the expression of the DElncRNAs. The upper and right edges are histograms of gene expressions.

vascular invasion, neoplasm histologic grade, and pathologic stage in TCGA database were analyzed. The results revealed that 3 miRNA, and 4 mRNA were associated with vascular invasion; 7 lncRNA, 1 miRNA, and 3 mRNA were associated with neoplasm histologic grade; 8 lncRNA, 1 miRNA, and 4 mRNA were associated with pathologic stage ($P < 0.05$; **Figures 8–10**).

DISCUSSION

CCA is one of the most common cancers worldwide, and its incidence and mortality remain high (26). Although great progress has been made in the treatment, prognosis and diagnosis of CCA in recent studies, CCA mortality remains high, which may be due to the lack of efficient biomarkers and the unclear mechanisms underlying CCA (1). Recent studies have demonstrated the vital role lncRNAs play in tumor progression, and some of these lncRNAs may be potential biomarkers for better prognosis and diagnosis (27). lncRNAs function in many ways. The ceRNA hypothesis postulates that lncRNAs may compete with miRNAs for the binding sites of miRNAs and may further affect mRNA expression through MREs (7). This hypothesis integrates the relationship among lncRNAs, mRNAs, and miRNAs and better explains the interaction among a variety of types of RNAs at the genetic level. We established a

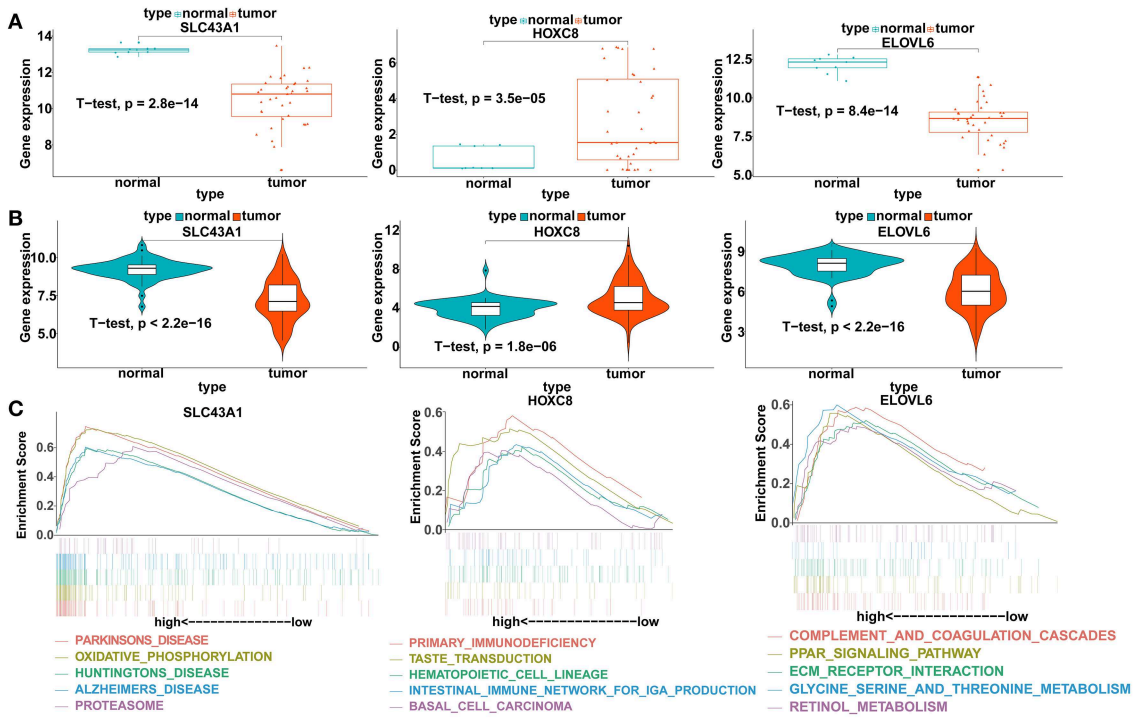

FIGURE 6 | Validation of the expression levels and gene set enrichment analysis (GSEA) of SLC43A1, HOXC8, and ELOVL6. **(A)** Scatter plots of mRNA levels of SLC43A1, HOXC8, and ELOVL6 in the TCGA training dataset. **(B)** Violin plots of mRNA levels of SLC43A1, HOXC8, and ELOVL6 in the GSE26566 validation dataset. **(C)** The top five functional gene sets enriched in the CCA samples are listed, which showed high expression of SLC43A1, HOXC8, and ELOVL6.

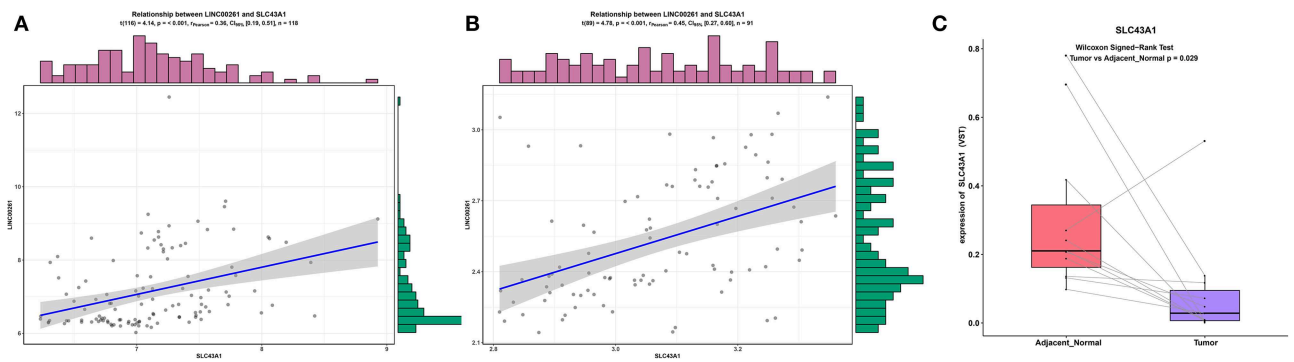

FIGURE 7 | Validation of the relationship between LINC00261 and SLC43A1. Regression analysis between the expression levels of LINC00261 and SLC43A1 in the GSE89749 **(A)** and GSE76297 **(B)** validation datasets. Violin plots of mRNA levels of SLC43A1 in the PUMCH validation dataset by using RT-PCR **(C)**.

lncRNA-miRNA-mRNA ceRNA network mediated by lncRNA at the transcriptome-wide level in this study to lay a useful foundation for studying the regulatory function of ceRNA in CCA.

To our knowledge, few studies have focused on predicting CCA prognosis, and no systematic examination has been performed on the prognostic value of ceRNA in CCA. In addition, rare reliable lncRNAs, miRNAs and mRNAs in relation to CCA can be treated as molecular biomarkers for detecting CCA and stratifying CCA risk. Under the background, the lncRNAs, mRNA, and miRNA expression data of 36 CCA samples and 9 normal samples were attained from the TCGA database. Based on the comprehensive integration of the lncRNA, mRNA and miRNA expression data of 36 CCA tissues and 9 normal tissues, a ceRNA network related to lncRNA was established to study the regulatory mechanism of ceRNA. Although CCA is characterized by extremely complicated biogenesis, DEGs can represent the complicity of tumor development as well as metastasis dissemination to a large extent; therefore, we used DEGs to establish the ceRNA

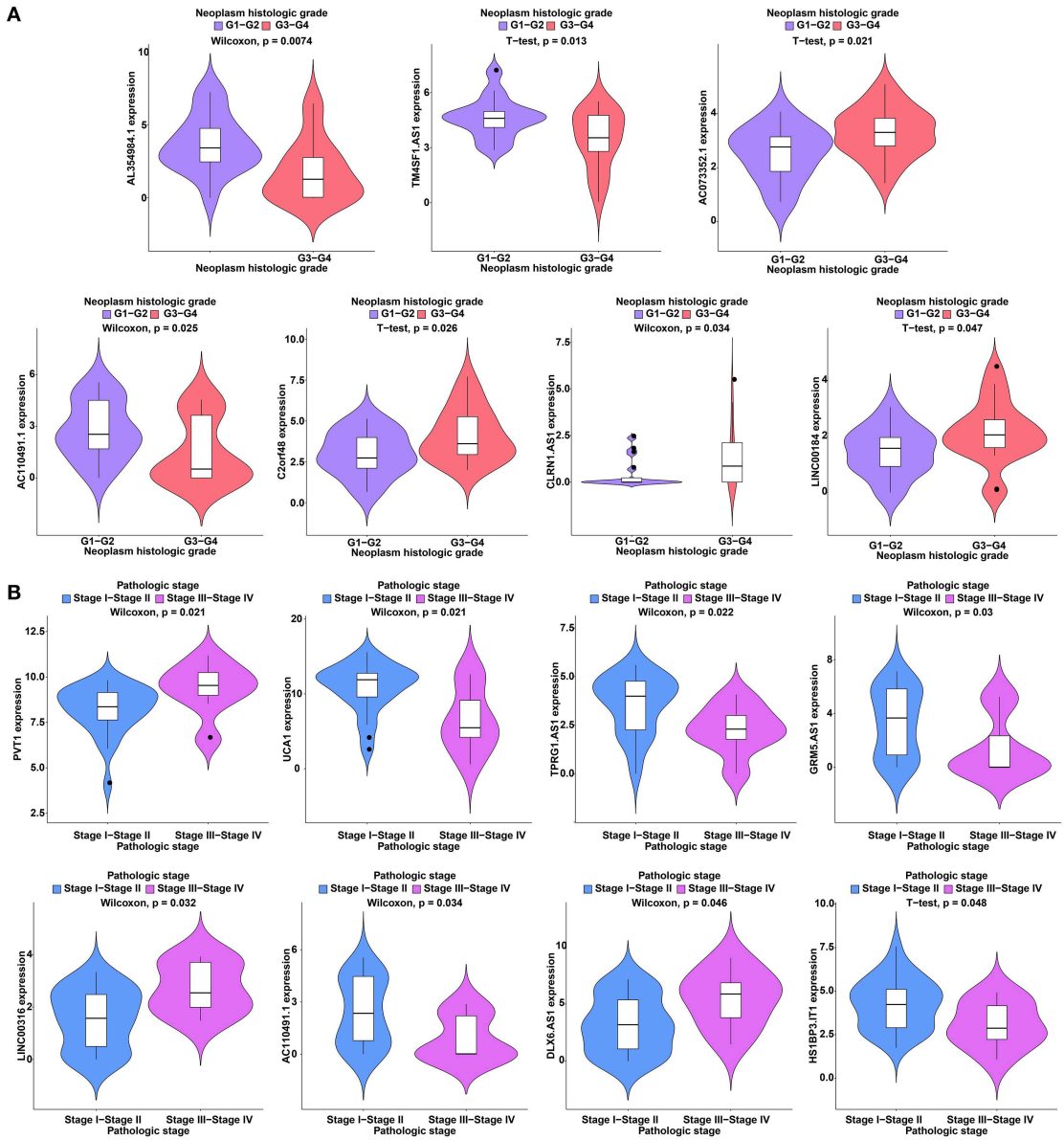

FIGURE 8 | Clinical feature analysis of DElncRNAs in the ceRNA network. The correlation between DElncRNAs involved in the ceRNA network and neoplasm histologic grade **(A)** and pathologic stage **(B)**.

network. In this study, we found 1,410 DElncRNAs in the CCA samples compared with the normal liver samples and 117 DElncRNAs targeting DEmiRNAs in the ceRNA network. Twenty-seven of them presented an obvious relevance to overall survival. There were 64 DEmiRNAs in the CCA tissues compared with the non-cancerous tissues and 14 DEmiRNAs targeted by DElncRNAs in the ceRNA network. Thereinto, 3 DEmiRNAs exhibited remarkable relevance to overall survival. We also found 3,494 DEmRNAs in the CCA tissues compared with the non-cancerous tissues, and 60 DEmRNAs targeted by DEmiRNAs were included in the ceRNA network. Thereinto, 13 DEmiRNAs presented a remarkable relevance to overall survival. The genes associated with overall survival are likely to indicate prognosis for CCA.

We analyzed the functions and pathways of DEmRNAs participating in the ceRNA network by using GO and KEGG. These DEmRNAs were mainly enriched in functions of "sequence-specific DNA binding," "sensory organ development" and "growth factor activity" and in the pathways of "endocrine resistance," "estrogen signaling pathway," and "TNF signaling pathway," etc., which are closely associated with tumorigenesis.

In the ceRNA network, hsa-mir-211 could interact with the majority of DElncRNAs and DEmRNAs (connection degree = 50), suggesting that hsa-miR-211 has

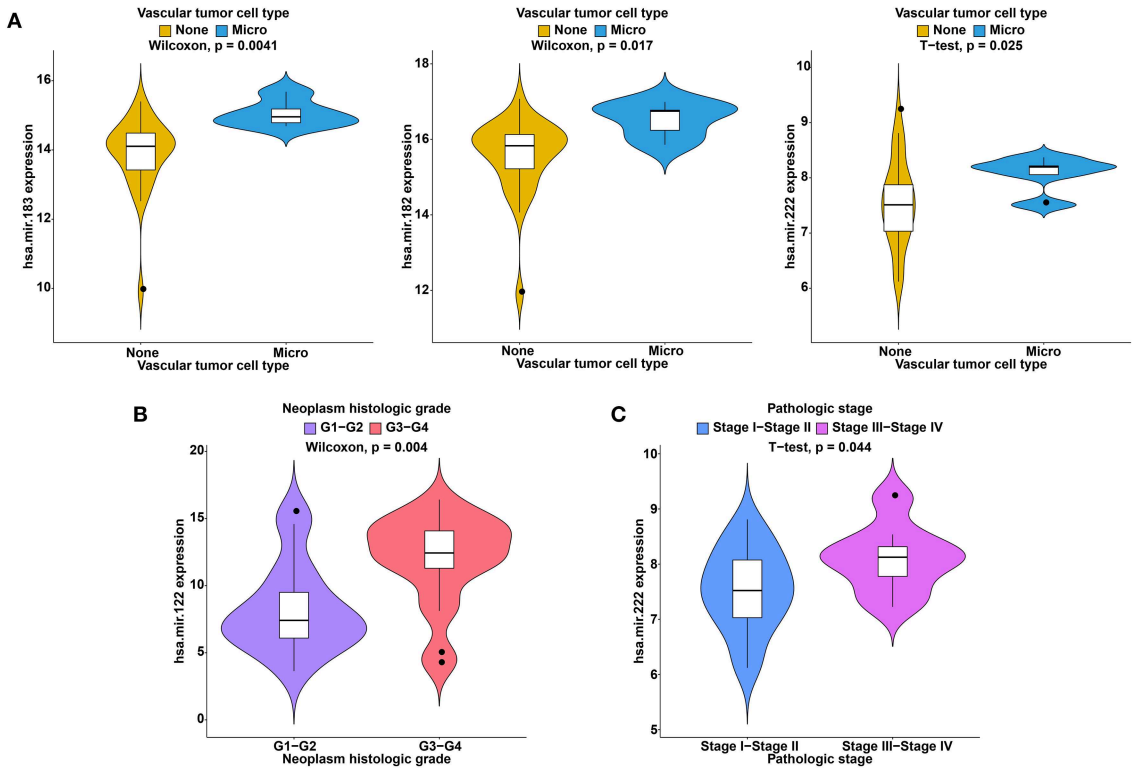

FIGURE 9 | Clinical feature analysis of DEmiRNAs in the ceRNA network. The correlation between DEmiRNAs involved in the ceRNA network and vascular invasion **(A)**, neoplasm histologic grade **(B)** and pathologic stage **(C)**.

a significant effect on the pathogenesis and prognosis of CCA. Although dysregulation of miR-211 is observed in many cancers, the function of miR-211 in CCA remains unclear. Computational methods were used to predict miR-211-5p by using conservation with mouse and Fugu rubripes sequences (28). The sequence is mapped to human chromosome 15, and it has been shown that miR-211-5p greatly impacts many types of cancers, such as colorectal cancer (CRC), non-small cell lung cancer (NSCLC), gastric cancer (GC), HCC, etc. (29–32). Based on studies conducted in the past, miR-211-5p was downregulated in GC, HCC and ovarian cancer (29, 30, 33), yet it was overexpressed in NSCLC (32). MiR-211 presented a decreasing trend in HCC samples compared with surrounding non-cancerous samples (29). The overexpression of miR-211 subdued the invasion and proliferation in SMMC7721 and HepG2 cells (29). MiR-211 can downregulate SATB2, leading to subdued HCC (29). MiR-211 also showed an obvious downregulation in GC (30). The invasion and proliferation of GC cells *in vitro* could be subdued by the overexpression of miR-211 and could be facilitated by downregulating the expression of miR-211 (30). The proliferation and invasion of GC cells could be partially inhibited by miR-211 by virtue of downregulating SOX4 (30). In contrast, miR-211 expression was upregulated in cell lines and tissues of human NSCLC (32). As miR-211 was overexpressed, the proliferation, colony formation, and invasion of NSCLC cells were enhanced (32). In addition, by targeting SRCIN1, miR-211 played a role in facilitating the proliferation of NSCLC (32). It was found in our study that miR-211 was downregulated in CCA tissues in comparison with normal liver tissues. In addition, a correlation analysis between DElncRNAs and DEmRNAs targeted by miR-211 was performed and revealed that some DElncRNAs and some DEmRNAs targeted by miR-211 have very strong correlations; among them, LINC00261 has the strongest correlation with SLC43A1 ($P = 1.58E{-}05$).

LncRNA LINC00261 is mapped to 20p11.21 and has been shown to be downregulated in many cancer tissues compared with their surrounding non-cancerous samples (34, 35). There was an obvious downregulation of LINC00261 in GC tissues and a negative relationship between its expression and advanced tumor status, clinical stage and unfavorable prognosis of patients with GC (36). LINC00261 plays a role as a tumor suppressor in GC because it weakens the stability of Slug proteins and subdues the EMT (36). The expression of LINC00261 in HCC tissues appears to be lower than that in the surrounding normal tissues (37). The decrease in LINC00261 expression was correlated with greater tumor size, higher TNM stage (III-IV), and worse survival in patients with HCC (37). Based on the functional assays, as LINC00261 is overexpressed in HCC cells, the proliferation, colony formation and invasion of cells and the EMT process were subdued *in vitro*. Additionally, when LINC00261 was upregulated, the expression levels of Notch1 and Hes-1 in HCC cells were downregulated; thus, Notch signaling was greatly

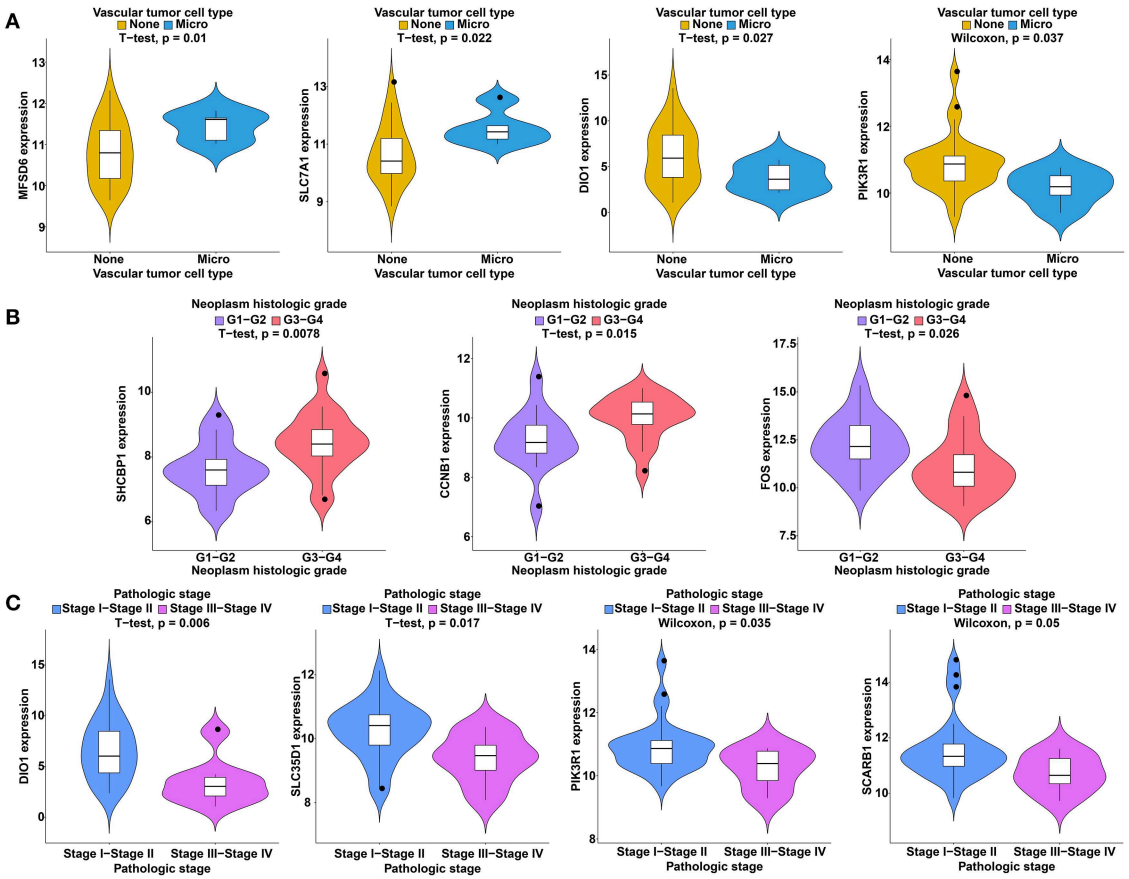

FIGURE 10 | Clinical feature analysis of DEmRNAs in the ceRNA network. The correlation between DEmRNAs involved in the ceRNA network and vascular invasion **(A)**, neoplasm histologic grade **(B)** and pathologic stage **(C)**.

reduced (37). LINC00261 expression levels in NSCLC tissues were suppressed compared with those in surrounding non-cancerous lung tissues (38). Low expression of LINC00261 was found to significantly related to TNM stage, lymph node status, and distant metastasis (38). Based on Kaplan-Meier analysis, low LINC00261 expression levels were largely indicative of poorer overall survival of patients with NSCLC (38). In our findings, LINC00261 was strongly correlated with SLC43A1. Furthermore, LINC00261 and SLC43A1 were both downregulated in CCA. Based on our results, we believe that lncRNA LINC00261 can mediate SLC43A1 expression by competing with miR-211 to participate in the progression of CCA.

Our analysis has great importance in that it reveals a ceRNA network in CCA, and some of the results were confirmed by another database. However, there were still several limitations in our study. First, the number of tumor tissue samples for the survival analysis was not large, and therefore, a larger CCA cohort is needed to find more ceRNAs associated with prognosis. Second, further study is warranted on the functions of key ceRNAs *in vitro* and *in vivo*. Third, Xu et al. reported that lncRNA SPRY4-IT1 functioned as a molecular sponge for miR-101-3p, antagonizing its ability to repress EZH2 protein translation in CCA (39). However, lncRNA SPRY4-IT1 does not exist in the TCGA database, so lncRNA SPRY4-IT1/miR-101-3p does not exist in our ceRNA network. In the future, with the emergence of better sequencing methods, our ceRNA network will be improved. Additionally, Zhang reported that lncRNA LINC01296 promotes cancer growth and progression by sponging miR-5095 in cholangiocarcinoma. However, miR-5095 is not a differentially expressed gene in the TCGA database. In general, gene expression data can be analyzed at three levels: the first level is the single gene level, which is the difference between the two experimental conditions; the second level is the analysis of functionally similar genes and interactions between genes; and the third level is research based on gene and protein networks (40). The most critical step is screening for potential DEGs from the expression profile (41). Our ceRNA network was established based on differentially expressed lncRNAs, miRNAs, and mRNAs; thus, the genes in our ceRNA network were more statistically and biologically significant. In our study, to identify the most significant interactions in the ceRNA of CCA and to cover as many interactions as possible, we set the thresholds for screening DEGs uniformly to FDR <0.05

and |log FC| >1. Although the lncRNA SPRY4-IT1/miR-101-3p and LINC01296/miR-5095 interactions were not included in the current ceRNA network, this deficiency did not impede the reliability of the ceRNA network, because our network was based on rigorous processes. First, we only included cancer-specific lncRNAs, miRNAs, and mRNAs with thresholds of FDR <0.05 and |log FC| >1. Second, the interactions between the DElncRNAs and DEmiRNAs and between the DEmiRNAs and DEmRNAs were predicted by experiment-supported databases, such as miRTarBase. These two methods ensured that the identified interactions would not only occur in computer simulations, but also based on evidence from experimental support. Therefore, we believe that the genes in the current ceRNA network are more reliable. In the future, with the emergence of better algorithms, better databases, and larger sample sizes, a more comprehensive ceRNA network will be established.

In conclusion, we built a ceRNA network mediated by dysregulated lncRNA in CCA on the basis of the "ceRNA hypothesis" through which the gene regulation mediated by lncRNAs in CCA can be comprehensively analyzed at the system level. This study provides a better understanding of the regulatory mechanism of ceRNAs mediated by lncRNA in CCA. Additionally, novel lncRNAs, miRNAs and mRNAs could be useful candidate diagnostic biomarkers or serve as potential therapeutic targets.

DATA AVAILABILITY

The datasets analyzed in this study are available in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) public repository.

ETHICS STATEMENT

This research project was approved by the Ethics Committee of Peking Union Medical College Hospital. Written consents were obtained from each patient.

AUTHOR CONTRIBUTIONS

HZhao and SC were the principal investigators who designed and conceived the study and obtained financial support. JLo, JX, and YB analyzed the data and wrote the manuscript. JM, JLi, WX, and HZhang prepared the dataset. All authors have read, revised, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00649/full#supplementary-material

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REVIEW
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Small Nucleolar RNAs: Insight Into Their Function in Cancer

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Small nucleolar RNAs (SnoRNAs) are a class of non-coding RNAs divided into two classes: C/D box snoRNAs and H/ACA box snoRNAs. The canonical function of C/D box and H/ACA box snoRNAs are 2'-*O*-ribose methylation and pseudouridylation of ribosomal RNAs (rRNAs), respectively. Emerging evidence has demonstrated that snoRNAs are involved in various physiological and pathological cellular processes. Mutations and aberrant expression of snoRNAs have been reported in cell transformation, tumorigenesis, and metastasis, indicating that snoRNAs may serve as biomarkers and/or therapeutic targets of cancer. Hence, further study of the functions and underlying mechanism of snoRNAs is valuable. In this review, we summarize the biogenesis and functions of snoRNAs, as well as the association of snoRNAs in different types of cancers and their potential roles in cancer diagnosis and therapy.

Keywords: small nucleolar RNA, cancer, rRNA processing, mRNA splicing, sdRNA, biomarker, therapy

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INTRODUCTION

Small nucleolar RNAs (snoRNAs) are an extensively studied non-coding RNAs that primarily accumulate in the nucleoli and consist of 60–300 nucleotides (NTs). SnoRNAs are mostly responsible for the posttranscriptional modification and maturation of ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and other cellular RNAs. SnoRNAs are divided into two classes: C/D box snoRNAs and H/ACA box snoRNAs. C/D box snoRNAs guide−2′-*O*-ribose methylation, and H/ACA box snoRNAs direct the pseudouridylation of NTs (1–4).

The box C/D family of snoRNAs is characterized by a kink-turn (k-turn) (stem–bulge–stem) structure and contains two conserved sequence elements: box C (RUGAUGA) and box D (CUGA) located at the 5′ and 3′ ends of the RNA molecule, respectively (**Figure 1**) (1). The k-turn motif is essential to the assembly of a small nucleolar ribonucleoprotein (snoRNP) including fibrillarin, Nop56, Nop58, and 15.5kD (3, 5, 6). Fibrillarin is an enzyme that is responsible for substrate methylation. Nop56, Nop58, and 15.5kD contribute to maturation, stability, and localization of snoRNAs. There are a few C/D box snoRNAs that do not form canonical snoRNPs to function in RNA methylation (7, 8). Most C/D box snoRNAs contain less conserved copies of box C and box D in the central region of the snoRNA, identified as box C' and box D', respectively, which generally carry one or two base modifications. The upstream elements of the box D/D′ motifs are complementary to target RNAs, allowing for alignment and methylation of appropriate NTs (3, 9, 10).

H/ACA box snoRNAs are composed of conserved box H and box ACA motifs (**Figure 1**). Box H is an ANANNA sequence (N represents any NT), and ACA box is a trinucleotide (ACA). H/ACA

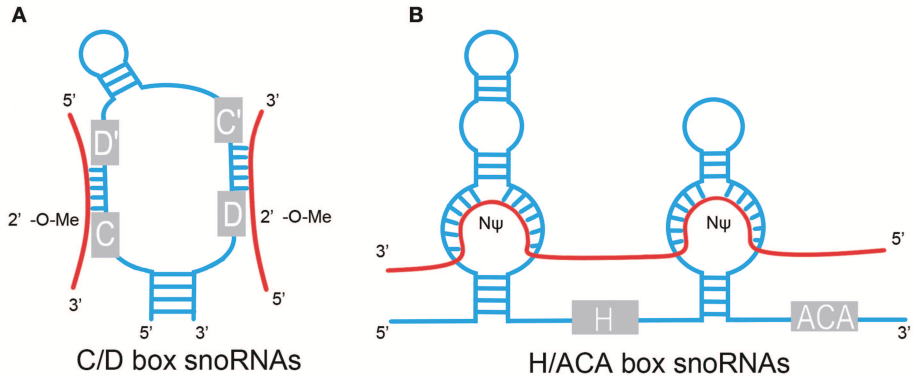

FIGURE 1 | Structural features of C/D box and H/ACA box snoRNAs. **(A)** C/D box. snoRNAs contain two conserved sequence elements, named box C (RUGAUGA), and box D (CUGA). Box C and box D are close to each other by the base pairing of the 5′ and 3′ termini and fold into a k-turn motif. The antisense elements, upstream of the box D/D′ motifs, are complementary to target RNAs and catalyze site-specific 2'-O-methylation (2′-O-Me) of the NT in target RNAs. **(B)** H/ACA box snoRNAs contain evolutionarily conserved structural elements, including box H (ANANNA), box ACA motif, and two pseudouridylation pockets. Pseudouridylation pockets are complementary to the substrate RNAs and responsible for pseudouridylation (NΨ). Red line, target RNAs.

box snoRNAs are marked by the "hairpin–hinge–hairpin–tail" secondary structure with box H in the hinge region and box ACA on the 3′ end of the last hairpin. An internal loop is located in the hairpin of H/ACA box snoRNAs with a 9–13 NT sequence on each strand complementary to the substrate RNAs, which forms a so-called pseudouridylation pocket. The distance between the site responsible for pseudouridylation and the H/ACA box is 14–16 NTs (3, 6, 9, 11). Similar to the C/D box snoRNAs, a set of core proteins are correlated with the H/ACA box snoRNAs to form stable and functional snoRNPs, including NHP2, NOP10, GAR1, and the pseudouridine synthase dyskerin (8).

THE BIOGENESIS OF SNORNAS

In vertebrates, most snoRNAs are encoded in the introns of protein-coding or non-coding genes, except for a small number of snoRNAs transcribed autonomously by RNA polymerase II (3). The biogenesis of most intronic snoRNAs includes cotranscription with the host gene, splicing, debranching of the intron lariat, and exonucleolytic digestion in the nucleoplasm. Recruitment of ribonucleoproteins to the nascent intronic snoRNAs is essential to the maturation of snoRNAs; this process is cotranscriptionally initiated. These proteins are critical to both processing stability and nucleolar localization (3, 12). In addition, many auxiliary factors are involved in snoRNP assembly and maturation, such as Shq1, Naf1, and NUFLP. SnoRNPs are transported to Cajal bodies, where they perform additional processing and maturation tasks (13). Thereafter, they are delivered to the nucleolus.

THE FUNCTIONS OF SNORNAS

Participation in rRNA Processing

One well-studied function of snoRNAs is their role in the modification, maturation, and stabilization of rRNA (3). As the research intensifies, hundreds of 2′-*O*-methylation or pseudouridylation residues located within conserved and functional regions of rRNAs have been found. A sequence upstream of box D and/or box D' recognizes target RNAs and results in the 2′-*O*-methylation of the fifth NT by the methylase fibrillarin (7, 14, 15). The conversion of uridines to pseudouridine is carried out by dyskerin, and the site destined for pseudouridylation is located 14–16 NTs upstream of box H and/or box ACA (9, 16, 17).

In addition to the rRNA modification function, some snoRNAs act in on pre-rRNA cleavage (18, 19). For example, SNORD3 promotes the proper pre-rRNA formation for subsequent endonucleotic processing (20–23). Furthermore, SNORD118, SNORD14, SNORD22, SNORA71, and probably, SNORD13 are involved in pre-rRNA cleavage (24–27).

Regulation of mRNA Splicing and Editing

Recent evidence indicates that snoRNAs play an important role in the regulation of gene expression. SNORD115 is encoded in the imprinted SNURF–SNRPN locus on human chromosome 15, which is frequently deleted in Prader–Willi syndrome (PWS) (28–30). SNORD115 contains an 18-NT sequence complementary to the alternative exon Vb of the serotonin receptor subtype 2C (5-HT2cR) and regulates the alternative splicing of 5-HT2cR (31). Besides the regulation of alternative splicing, SNORD115 influences RNA editing. The site-specific adenosine-to-inosine (A-to-I) base conversion is essential to the generation of 5-HT2cR (32). Vitali et al. demonstrated that SNORD115 inhibited the efficiency of the ADAR2-mediated RNA editing of 5-HT2cR by forming a bona fide snoRNP particle (33). Moreover, using bioinformatic prediction and experimental verification, Rishore et al. identified that SNORD115 regulated the alternative splicing of five pre-mRNAs (DPM2, TAF1, RALGPS1, PBRM1, and CRHR1). They demonstrated that SNORD115 generated shorter RNAs, called processed small nucleolar RNAs (psnoRNAs), that directed the regulation of alternative splicing (34). Two gene clusters

encoding SNORD115 and SNORD116 are located in the imprinted locus 15q11-q13 containing 47 repeats of SNORD115 along with 27 copies of SNORD116. Using genome-wide array analysis after overexpressing SNORD115 and SNORD116 in human embryonic kidney (HEK) 293T cells, Falaleeva et al. found that SNORD115 and SNORD116 influenced the expression levels of over 200 genes and modified each other's activity (35). Bazeley et al. found that energetically favorable putative targets of SNORD116 were correlated with exons that were capable of alternative splicing and speculated that SNORD116 was involved in the regulation of alternative splicing (36). Wu et al. identified that 5′ snoRNA-capped and 3′ polyadenylated lncRNA (SPA) required snoRNP complexes to protect them from trimming by 5′-3′ exoribonuclease 2 (XRN2). The most well-understood function of SNORD27 is to guide the methylation of rRNA. Recent research demonstrated that SNORD27 regulated the alternative splicing of the transcription factor E2F7 pre-mRNA by directing RNA–RNA interaction (7). A similar function was reported for SNORD88C, which produced small RNAs derived from snoRNAs (sdRNAs) containing the box C' that was complementary to several pre-mRNAs including FGFR3 and regulated the alternative splicing of FGFR3 pre-mRNA (37). In another study, Huang et al. discovered that SNORA50A inhibited mRNA 3' processing by blocking the Fib1-poly(A) site interaction, which was the first report that snoRNA regulated mRNA 3' processing (38).

Involvement in Stress Response and Metabolic Homeostasis

Michel et al. found that three snoRNAs (SNORD32A, 33, and 35A) encoded in the ribosomal protein L13a (Rpl13a) locus were increased significantly under oxidative stress induced by palmitate and hydrogen peroxide treatment. Knockdown of these three snoRNAs simultaneously increased resistance against palmitate *in vivo* (39). Furthermore, snoRNA ACA11 has been found to suppress oxidative stress by downregulating ribosomal protein genes and other snoRNAs (40). Brandis et al. identified that the loss of snoRNA U60 reduced plasma membrane-to-endoplasmic reticulum cholesterol trafficking and increased *de novo* cholesterol synthesis. This finding suggested that U60 played a function in regulating intracellular cholesterol trafficking (41). Other reports demonstrated that snoRNAs regulate cellular metabolic homeostasis. For example, snoRNA U17 regulated cellular cholesterol trafficking *via* the encoding of hypoxia-upregulated mitochondrial movement regulator (HUMMR) by its target mRNA. Four snoRNAs encoded in Rpl13a, snoRNAs U32A, U33, U34A, and U35A, regulated systemic glucose metabolism (42, 43).

The Role and Molecular Mechanisms of snoRNAs in Cancer

SnoRNAs are widely involved in many physiological and pathological processes; indeed, emerging evidence suggests that snoRNAs have tumor-suppressive or oncogenic functions in various cancer types (**Table 1**). SnoRNAs are reported to participate in many biological cancer processes, including the invasion of growth suppressors and cell death, activation of invasion and metastasis, and angiogenesis and sustained proliferative signaling. The underlying molecular mechanisms are diverse.

SnoRNAs and the P53 Regulatory Pathway in Cancer

P53 is a well-known tumor suppressor that responds to diverse cellular stresses to regulate the expression of target genes involved in cell cycle arrest, apoptosis, and DNA repair (73). Increasing evidence suggests that snoRNAs are closely associated with the p53 regulatory pathway. For instance, a recent study identified that snoRNAs and FBL were usually overexpressed in human breast and prostate cancers and that this overexpression promoted tumorigenicity *in vitro* and *in vivo* (74). Further research found that oncogene Myc upregulated the expression level of FBL and led to elevated snoRNA biogenesis, inducing p53 suppression. Cellular stress induced by knockdown of snoRNA pathway genes increased the accumulation of p53 by promoting the binding of ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) with MDM2 and led to p53 stabilization. Furthermore, impaired snoRNA biogenesis induced by FBL depletion promoted PTB binding to the p53 internal ribosome entering site (IRES) and regulated the initiation of p53 translation (74). A similar finding has been reported by Langhendries et al. U3 and U8 were upregulated in breast cancer, and depletion of U3 and U8 inhibited tumorigenicity of breast cancer cells *in vivo* and *in vitro* (50). Depletion of U3 or U8 led to ribosome biogenesis dysfunction by inhibiting pre-rRNA processing and reduced production of mature rRNAs (50). SnoRNA42 was overexpressed in non-small cell lung cancer (NSCLC) and played an oncogenic role by regulating the expression of p53. Doxorubicin-induced DNA damage induced the expression of the GAS5-derived snoRNAs (U44 and U77) in a p53-dependent manner. Chromatin immunoprecipitation sequencing (ChIP-seq) experiments confirmed that p53 directly controlled GAS5 transcription. GAS5-derived snoRNAs are closely correlated with p53 levels in colorectal tissue, suggesting that GAS5-derived snoRNAs have a critical role in p53-associated signaling pathways in colorectal cancer (CRC) (75).

There are several other snoRNAs associated with the p53 regulatory pathway. In a multistage germline copy number variation (CNV)-based genome-wide association studies (GWAS), a low-frequency duplication at 15q13.3 containing SNORA18L5 was strongly correlated to risk of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Further studies showed that SNORA18L5 was overexpressed in HCC tissues compared with adjacent normal tissues and played an oncogenic role in HCC (57). Overexpression of snoRNA18L5 kept RPL5 and RPL11 from binding to MDM2, resulting in increased MDM2-mediated ubiquitination degradation of p53 (57). It is well known that p53 mutations in cancers often exert oncogenic gain-of-function properties that contribute to tumorigenicity. Increasing evidence shows that in addition to wild-type p53, mutant p53 also interacts with snoRNAs in cancers (76). For instance, Pourebrahim et al. identified that p53-mutant mice

TABLE 1 | SnoRNAs involved in human cancers.

snoRNAs	Class	Expression level	Function	Cancer	Intersection molecules and/or pathway	Reference
SONRA42/MBI-43	H/ACA box	Increased	OG	NSCLC, CRC	P53	(44, 45)
SNORD78/U78	C/D box	Increased	OG	NSCLC, PCa		(46)
U50/SNORD50A/RNU50	C/D box	Decreased	TS	BRCA, PCa	Ras-ERK1/ERK2	(47–49)
U3/SNORD3@	C/D box	Increased	OG	BRCA	p53	(50)
U8/SNORD118/LCC	C/D box	Increased	OG	BRCA	p53	(50)
RNU44/SNORD44/U44	C/D box	Decreased	TS	BRCA		(51)
RNU43/SNORD43/U43	C/D box	Decreased	TS	BRCA		(51)
SNORD48/U48/RNU48	C/D box	Decreased	TS	BRCA		(51, 52)
SNORD29/U29/RNU29	C/D box		OG	BRCA	YB-1	(52)
SNORD34/U34/RNU34	C/D box		OG	BRCA	YB-1	(52)
SNORD67/HBII-166	C/D box		OG	BRCA	YB-1	(52)
SNORD33/U33/RNU33	C/D box		OG	BRCA	YB-1	(52)
ACA44/SNORA44	H/ACA box		OG	BRCA	YB-1	(52)
SNORD114-1/14q(II-1)	C/D box	Increased	OG	APL	Rb/p16	(53)
SNORD112-114	C/D box	Increased	OG	APL		(53)
SNORD35A/RNU35/RNU35A/U35	C/D box	Increased	OG	AML	AES and DDX21	(54)
SNORD74/U74/Z18	C/D box	Increased	OG	AML	AES and DDX21	(54)
SNORD14D	C/D box	Increased	OG	AML	AES and DDX21	(54)
SNORD43/RNU43/U43	C/D box	Increased	OG	AML	AES and DDX21	(54)
SNORA21/ACA21	H/ACA box	Increased	OG	CRC		(55)
SNORA15/ACA15A	H/ACA box	Increased	OG	CRC		(56)
SNORA41/ACA41A	H/ACA box	Increased	OG	CRC		(56)
SNORD33/U33/RNU33	C/D box	Decreased	TS	CRC		(56)
SNORA18L5	H/ACA box	Increased	OG	HCC	stress-RPs-MDM2-P53	(57)
SNORD126/MIR1201/MIRN1201	C/D box	Increased	OG	HCC	PI3K–AKT	(58)
ACA11/SCARNA22	H/ACA box	Increased	OG	HCC, MM	PI3K–AKT, RP genes	(40, 59)
SNORD76/U76	C/D box	Increased /Decreased	OG/TS	HCC,GBM	Wnt/-catenin pathway	(60, 61)
SNORA47/HBI-115	H/ACA box	Increased	OG	HCC		(62)
SNORD113-1/14q(I-1)	C/D box	Decreased	TS	HCC	ERK1/2 and SMAD2/3	(63)
SNORA55/ACA55	H/ACA box	Increased	OG	PCa		(64)
SNORD59A/U59/RNU59	C/D box	Decreased	TS	PCa		(65)
SNORD82/RNU82/U82/Z25	C/D box	Decreased	TS	PCa		(65)
SNORD116/HBII-85	C/D box	Decreased	TS	PCa		(65)
SNORD117/U83	C/D box	Decreased	TS	PCa		(65)
SNORD24/RNU24/U24	C/D box	Decreased	OG	ACC	miRNAs	(66)
SNORA74B/U19-2	H/ACA box	Decreased	TS	GBC	AKT/mTOR	(67)
SNORD47/RNU47/U47	C/D box	Decreased	TS	GBM		(68)
SNORD94/U94	C/D box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA70/DXS648E/RNU70/U70	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORD10/mgU6-77	C/D box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA13/ACA13	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA38/ACA38	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA79/ACA65A	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA46/ACA46	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SCARNA9/Z32/mgU2-19/30	SCARNA	Increased	OG	Osteosarcomas	Mutant p53, Est2	(69)
SNORA23/ACA23	H/ACA box	Increased	OG	PDAC	SYNE2	(70)
SNORD35B/RNU35B/U35B	C/D box	Decreased	TS	HNSCC		(71)
SNORD114-10/14q(II-10)	C/D box	Decreased	TS	OC		(72)

OG, oncogene; TS, tumor suppressor; BRCA, breast cancer; PCa, prostate cancer; APL, acute promyelocytic leukemia; MM, multiple myeloma; GBM, glioblastoma; ACC, adenocarcinoma; GBC, gallbladder cancer; PDAC, pancreatic ductal adenocarcinoma; HNSCC, head and neck squamous cell carcinoma; OC, ovarian cancer.

developed osteosarcomas with increased metastasis as compared with p53-null mice. Using comprehensive transcriptome RNA sequencing analysis of 16 tumors, they also found that a cluster of snoRNAs were upregulated in p53 mutant tumors. An analysis of regulatory elements showed that the Est2-binding motif was highly enriched in these deregulated snoRNAs. Genetic deletion of the Esr2 transcription factor reduced the expression of these snoRNAs in p53 mutant mice and abrogated the mutant p53 prometastatic phenotype (69).

SnoRNAs in the Regulation of Other Cancer-Related Signaling Pathways

There are many other signaling pathways associated with snoRNAs, including the phosphoinositide 3-kinase (PI3K)–AKT and Wnt/β-catenin pathways discussed here. The PI3K–AKT pathway is a highly conserved cell signaling system in most multicellular organisms, which is critical to diverse cellular processes such as stemness, cell proliferation, differentiation, and cell death (77). The role of the PI3K–AKT signaling pathway in cancer is well-documented (77). A number of snoRNAs have been implicated in PI3K–AKT signaling, either directly or indirectly. ACA11 knockdown significantly decreased the phosphorylation of PI3K and AKT, whereas ACA11 overexpression promoted HCC cell growth and metastasis by activating the PI3K–AKT pathway (59). In another study, SNORD126 activated PI3K–AKT signaling by upregulating FGFR2 and promoted HCC and CRC cell growth (58). PH domain leucine-rich repeat protein phosphatase (PHLPP) is an endogenous inhibitor of the AKT pathway (77). In gallbladder cancer, SNORA74B knockdown suppressed activation of the AKT/mechanistic target of rapamycin (mTOR) pathway *via* inducing PHLPP expression (67). In the Wnt/β-catenin pathway, the major pathway regulating the development and progression of HCC, SNORD76 knockdown significantly decreased the level of β-catenin, c-Myc, and cyclinD1. Conversely, overexpression of SNORD76 promoted HCC tumorigenicity through activation of the Wnt/β-catenin pathway (60).

SnoRNAs as the Precursors of Small RNAs

In 2008, Kawaji et al. performed an unbiased sequencing of human small RNAs (19–40 NTs) and found independent classes of small RNAs originating from noncoding RNAs (78). Since then, a wealth of sdRNAs have been identified. In 2009, Taft et al. performed a systematic analysis of small RNA by deep sequencing libraries from various eukaryotic tissues and found that small RNAs with evolutionarily conserved size and position were derived from a large proportion of snoRNA loci in animals (human, mouse, chicken, and fruit fly), *Arabidopsis*, and fission yeast (79). In recent years, the processing patterns of snoRNAs have raised substantial attention, and increasing evidence shows that these small RNAs have a role in cancers. SnoRNA–miR-28, an miRNA-like non-coding RNA derived from snoRNAs, was significantly upregulated in breast tumors and promoted proliferation of tumor cells (80). SNORD28, a p53-repressed snoRNA located in SNHG1, can be processed into smaller miRNA-like molecules that are capable of binding argonaute (AGO) and exerting miRNA-like effects. This procession can be repressed by p53, the underlying mechanism of which is unclear (80). At the same time, snoRNA–miR-28 acts as an miRNA and directly interacts with TAF9B's 3'-untranslated region (3'-UTR), leading to the reduction of TAF9B mRNA and protein expression levels. The reduced expression impairs the stability of p53 by promoting the binding of MDM2 to p53. Collectively, a regulatory loop exists between p53, SNHG1, snoRNA-miR-28, and TAF9B (80). miR-768-5p, another miRNA-like non-coding RNA derived from SNORD17, was reported to bind to YB-1 and may play a role in the development of cancers (52).

PIWI-interacting RNAs are identified as inhibitor of transposable elements (TEs) in the germline and play an important role in regulating target RNA to silence its expression *via* base-pairing recognition (81). In addition to miRNA-like functions, these sdRNAs also have the ability to play piRNA-like functions. Pi-sno-75, a piRNA derived from SNORD75 located in GAS5, can specifically bind to PIWIL1 and PIWIL4 (piRNA binding proteins in *Homo sapiens*) and bearing modification of $2'$-*O*-methylation at the $3'$ terminus (82). Microarray results showed that TRAIL, a tumor-specific suppressor, was upregulated by pi-sno-75 synthesized in breast cancer cells. ChIP results showed that overexpression of pi-sno-75 increased the H3K4me3 level and decreased the H3K27me3 level within −169 base pair (bp) of TRAIL promoter in a PIWIL1/PIWIL4-dependent manner. Further research identified the molecular mechanism of this regulation: the pi-sno-75/PIWIL complex can interact with WDR5 and recruit the entire hCOMPASS-like complex along with SMYD3 to the promoter region of the TRAIL gene (82). Pi-sno-75 exhibits antitumor activity *in vitro* and *in vivo* by upregulating the expression of TRAIL (82). Uzunova et al. identified that sdRNAs derived from SNORD44, SNORD74, SNORD78, and SNORD81 were upregulated in prostate cancer. In particular, the levels of, SNORD78 and its sdRNAs were obviously higher in patients with metastatic diseases (65).

The Role of snoRNAs in Cancer Stem Cells

Cancer stem cells have the capacities of self-renewal, differentiation, and tumorigenicity. The presence of cancer stem cells has been reported in various cancers, which may explain why current chemotherapies cannot consistently eradicate cancers (83). A growing body of research suggests that snoRNAs play an important role in cancer stem cells. Self-renewal activity is essential in leukemogenesis. Zhou et al. identified that amino-terminal enhancer of split (AES), C/D box snoRNAs, and rRNA $2'$-*O*-methylation are essential for the AML1–ETO–induced self-renewal of leukemia cells *in vitro* and *in vivo* (54). Knockdown of AES leads to reduction of snoRNAs (primarily C/D box snoRNAs) and suppression of translation efficiency in AE9a-transduced leukemia cells. DDX21 is an RNA helicase that binds to snoRNA/RNP to facilitate rRNA modification. The underlying mechanism involves the knockdown of AES, which reduces the association of DDX21 with the C/D box snoRNP complex including FBL, NOP598, NOP56, and NCL and followed by suppression of snoRNAs. C/D box snoRNAs are highly expressed in acute myeloid leukemia (AML) and correlated closely with the *in vivo* frequency of

leukemic stem cells. Knockdown of SNORD14D or SNORD35A suppressed the clonogenic potential of leukemia cells *in vitro* and delayed leukemogenesis *in vivo* (54). In previous studies, Mannoor et al. demonstrated that (aldehyde dehydrogenase 1) was a cancer stem cell marker, as $ALDH1^+$ cancer cells have extensive self-renewal, proliferation, and *in vivo* tumorigenic potentials. There are 22 snoRNAs that display differential expression in $ALDH^+$ cancer cells compared with $ALDH^-$ cancer cells (44). SNORA3 and SNORA43 overexpressed in lung cancer stem cells and inversely associated with survival in NSCLC patients. Along with CD133, another important maker for lung cancer stem cells, SNORA42 was confirmed to be especially dysregulated in lung cancer stem cells (44). Knockdown of SNORA42 inhibited self-renewal capacity and *in vitro* tumorigenesis by inducing apoptosis and reducing the transcript level of stem cell-associated genes including OCT4, Nanog, Sox2, Notch1, Smo, and ABCS2, suggesting SNORA42 may associate with the expression of core stem cell transcription factors in lung cancer stem cells (44). SNORD78 is also reported to be upregulated in cancer stem cells in NSCLC and is required for the self-renewal of cancer stem cells in NSCLC (46).

SnoRNAs as Biomarkers and Therapeutic Targets of Cancers

As mentioned above, aberration in the expression of snoRNAs was found to be prevalent in many cancers; some of these anomalies are cancer type specific. Substantial research has shown that many snoRNAs are stably expressed and detectable in body fluids including the blood plasma, serum, and urine of cancer patients. Their expression levels are closely associated with diagnosis, prognosis, and classification of subtypes. Given these specifics, snoRNAs have the potential to become cancer biomarkers (84, 85).

The clinical value of snoRNA expression analysis in the diagnosis of certain subtypes of peripheral T-cell lymphoma (PTCL) has been demonstrated. Moreover, Valleron et al. found that the overexpression of snoRNA HBI-239 and HBI-239-processed miRNA predicted good prognosis in angio-immunoblastic T-cell lymphoma (AITL) and PTCL not otherwise specified (PTCL-NOS) (86). Berquet et al. investigated the snoRNA expression profiles in B-cell chronic lymphocytic leukemia (CLL) patients and identified that immunoglobulin heavy chain variable region gene (IGHV)-mutant patients exhibiting overexpression of 20 snoRNAs had a shorter treatment-free survival (TFS) (87). SNORA70F and SNORD116-118 were developed into a 2-snoRNA model that appeared to distinguish two different prognostic CLL groups (88). Furthermore, a research group identified that snoRNA expression profiles can be used for the classification of leukemia subgroups due to the differential expression among these various groups (89). SNORD33, SNORD66, and SNORD76 were overexpressed in NSCLC patients and were detectable in plasma, yielding 81.1% sensitivity and 95.8% specificity in distinguishing NSCLC patients. SNORA42 was overexpressed in NSCLC patients; the levels were inversely correlated with the survival of patients, providing a potential marker for diagnosis and prognosis in NSCLC. SNORA47, SNORA68, and SNORA78 were reported to accurately predict overall survival of NSCLC patients in a training set of 77 cases, which may become an snoRNA-based model for predicting overall survival in lung cancer patients (44, 90, 91). SNORD42 and SNORD21 were also reported to be promising predictive biomarkers for prognosis in CRC patients (55, 92). The expression level of SNORA18L5 in HCC tissues was correlated with the survival time of patients; patients with high SNORA23 expression had a shorter disease-free survival time (57, 70). RUN44, RUN43, and RUN48 were downregulated in breast cancer and were associated with a poor prognosis. In a recent study, Krishnan et al. found a large number of snoRNAs that were promising prognostic markers for breast cancer (51, 93). SNORD114-10 was downregulated in metastatic omentum tissues, suggesting that this snoRNA may provide a prediagnostic biomarker for ovarian cancer metastasis (72). These studies strongly indicate that snoRNAs offer promising novel diagnostic and prognostic markers across a range of cancer types. However, further investigation is necessary, and many challenges must be overcome prior to their application in clinical settings.

Since snoRNAs participate in tumorigenesis, tumor aggressiveness, and staging, they are ideal candidates for cancer therapy. The regulation of snoRNA expression may contribute to the goal of cancer treatment. For example, antisense oligonucleotide (ASO)-mediated downregulation of SNORA23 expression reduced tumor growth, dissemination, and liver metastasis in pancreatic ductal adenocarcinoma (70). Our studies demonstrated that SNORD44 was downregulated in CRC and that overexpression of SNORD44 by an oncolytic adenovirus inhibited CRC growth (94). With the expansion of research and the further development of applicable technologies, snoRNAs may become major cancer therapeutic targets in the near future.

CONCLUSIONS AND PROSPECTS FOR FURTHER RESEARCH

Although the landscape of snoRNA function in its entirety remains still unclear, previous studies have confirmed the critical roles of snoRNAs in rRNA processing, gene transcription, RNA splicing, and other cellular processes. As sequencing and microarray analysis technologies evolve, more and more tumor-related snoRNAs are being discovered. Recent studies have demonstrated that aberrant expression and mutations in specific snoRNAs were associated with tumorigenesis and metastasis. The tissue- and cancer-specific expression of snoRNAs can be used as reliable prognostic markers for cancer diagnosis and novel therapeutic targets. However, the field is still in its infancy, and our understanding of the role of snoRNAs in tumors remains incomplete. Currently, the effect of snoRNA on cellular signaling pathways is also poorly understood. Functional screening of RNA interference libraries and/or clustered regularly interspaced short palindromic repeats (CRISPR)-based libraries is necessary to explore snoRNA functions in signaling pathways. SnoRNAs do not always act directly;

indeed, most of their actions are indirect binding to other molecules. The identification of more molecules that interact with snoRNAs using RNA precipitation combined with high-throughput mass spectrometry and RNA immunoprecipitation (RIP) technologies is promising. Moreover, animal models are important for understanding the function of non-coding RNA in tumorigenesis and development. However, animal models of snoRNAs in cancer remain insufficient to date. Furthermore, emerging evidence suggests that some non-coding RNAs contain translated open reading frames that are able to encode proteins. This hints at whether snoRNAs also have the ability to encode proteins and provides a whole new direction for investigating snoRNA function. A better grasp of the role of snoRNAs in tumors will help us to understand tumors more comprehensively and may offer not only novel diagnostic biomarkers but also effective therapeutic targets in the near future.

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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REVIEW
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Recent Progress on Circular RNA Research in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is a myeloid malignancy characterized by the proliferation of abnormal and immature myeloid blasts in the bone marrow. Circular RNA (circRNA) is a novel class of long non-coding RNA with a stable circular conformation that regulates various biological processes. The aberrant expression of circRNA and its impact on AML progression has been reported by a number of studies. Despite recent advances in circRNA research, our understanding of the leukemogenic mechanism of circRNA remains very limited, and translating the current circRNA-related research into clinical practice is challenging. This review provides an update on the functional roles of and research progress on circRNAs in AML with an emphasis on mechanistic insights. The challenges and opportunities associated with circRNA-based diagonostic and therapeutic development in AML are also outlined.

Keywords: acute myeloid leukemia (AML), circular RNA (circRNA), biomarker, apoptosis, cell proliferation, drug resistance, targeted therapy

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INTRODUCTION

Acute myeloid leukemia (AML) is the most common leukemia in adults, presenting great biological and clinical heterogeneity (1). In 2018, there were 19,520 new cases of AML in the United States (2), and, based on survey data, there were 14,100 reported cases in China in 2015 (3). Various genetic and epigenetic aberrations arrest hematopoietic cell differentiation and maturation events, leading to the accumulation of abnormal and immature hematopoietic progenitor cells in the bone marrow and peripheral blood. These abnormalities are often associated with lethal infection, organ infiltration, and cytopenias (4, 5).

Investigations on the genetic and molecular mechanisms of cancer have recently shifted from protein-coding genes to non-coding transcripts (6). About 98% of the human genome represents non-coding DNA sequences, and the majority of the human transcriptome is classified as non-coding RNAs (ncRNAs) (7). Circular RNA (circRNA) belongs to the family of endogenous ncRNA and has a non-polyadenylated closed single-stranded and continuous loop structure due to a covalent phosphodiester bond between the 3′ and 5′ ends (8). The size of circRNA ranges from a few hundred to thousands of nucleotides (9). Global circRNA expression profiling has revealed that they are abundant transcripts of well-regulated back-spliced RNA and that their biogenesis is regulated by cis-elements and/or trans-factors. The back-spliced RNA is produced as a result of back-splicing that ligates a splice donor site with an acceptor site present upstream and downstream, respectively, on the mRNA transcript, resulting in a covalently closed circRNA as well as an alternatively spliced linear RNA with skipped exon(s) (1). The circular and non-polyadenylated structure makes the circular RNA more stable. circRNAs exhibit evolutionary conservation across the eukaryotes

(10), and circRNA expression is tissue- and developmental stage-specific (11–13). Studies have shown that circRNAs regulate gene expression (14, 15) and are involved in regulating vital cellular events such as differentiation, proliferation, growth, signaling, and aging (11–13, 16, 17). Recently, aberrant expression of circRNAs has been implicated in the progression and pathogenesis of hematopoietic malignancies (18–20) and solid tumors (21–23). Numerous circRNAs with altered expression have been reported to be involved in leukemogenesis (24–26). miRNAs, short stretches of RNA (23 nt), are associated with various biological processes (2). circRNAs are also implicated in tumorigenesis, metastasis, and drug resistance (22). The most established mechanism of action of circRNAs is its "sponge" function via binding to miRNAs (14, 27), proteins (21, 28, 29), or DNA (10, 30). The most documented role of circRNAs in AML is modulating mRNA stability and translation by sequestering the mRNA transcript and protein (31, 32), although circRNA-RNA binding protein (RBP)/DNA interactions remain obscure and need further exploration.

This review compiles the roles of recently reported circRNAs that show altered expression in AML. Moreover, we also present the potential molecular mechanisms of certain circRNAs. Finally, we discuss the main challenges related to research on circRNA.

BIOGENESIS AND FUNCTIONS OF circRNA

The unique characteristic of circRNAs is associated with their production, which mostly occurs via the canonical and non-canonical splicing (3) of exons (4) and introns, non-coding antisense, 3′ and 5′ UTR or the intergenic region (4, 5). Non-canonical splicing is an unconventional mechanism of splicing characterized by the ligation of splice sites that are present at a distance from the currently annotated exons. The process aids in gene expression regulation and is important in evolution since it acts as a source of newly emerging transcripts (6).

Based on their origin of production and their determination by RNA-sequencing (RNA-seq), the circRNAs are classified into four types: exonic circRNA (ecircRNA) (4, 7), circular intronic RNA (ciRNA) (5), retained-intron or exon-intron circRNA (7, 8), and intergenic circRNA (4). Two models of circularization, illustrated in **Figure 1**, explain circRNA biogenesis via lariat-driven circularization (exon skipping) (**Figure 1B**) (9) or intron pairing-driven circularization (**Figure 1C**) (9). circRNA biogenesis is regulated by both *cis*- and *trans*-acting factors (33), with the latter also known as RNA binding proteins (RBP) (**Figure 1D**) (10). Some circRNAs, in which the introns are not trimmed and are retained in exons, are known as exon-intron circRNAs or EIciRNAs (**Figure 1D**) (8). Unique splicing mechanisms may also result in the generation of novel circRNAs known as ciRNA (intronic circRNA) (5) (**Figure 1E**) and tRNA intronic circular RNAs (tricRNAs) (**Figure 1F**) (11).

The most established function of circRNAs is through sponge formation with miRNAs and proteins (**Figure 1H**). In the case of circRNA-miRNA sponge formation, the sequestration of miRNA by the circRNA allows the binding of translational machinery to the specific mRNA, thus causing gene derepression. Increased expression of genes that, for example, are associated with migration, differentiation, and proliferation, may contribute to leukemogenesis (10). Direct RBP sponging (circRNA-RBP) (**Figure 1I**) or indirect circRNA-miRNA-RBP interactions by circRNAs can also induce leukemogenesis, because RBPs are also implicated in cell cycle progression and the biogenesis of circRNA, ultimately thereby affecting cell differentiation, proliferation, and apoptosis (12).

Although circRNAs contain an open reading frame (ORF), they generally lack the essential components necessary for translation, such as a poly(A) tail and a 7-methylguanosine cap. Nevertheless, mounting evidence suggests the translation ability of circRNAs (**Figure 1J**) (13). For example, an RNA modification motif, N6-methyladenosine (m6A), enriched in circRNAs, facilitates the translation of circRNA in human cells (14). Overall, the translational mechanisms of circRNAs are not well-established and need further investigation.

circRNAs IN AML

Circular RNAs as Potential Diagnostic and Prognostic Biomarkers (Table 1)

Various molecular-based biomarkers (e.g., cytogenetics, genetics, epigenetics, proteomics, and miRNA) have been reported in AML and have been comprehensively reviewed (15). circRNAs are emerging as potential biomarkers in the diagnosis and treatment of AML (**Figure 2A**) due to their stability against exonucleolytic degradation, tissue-specific expression patterns, and abundance in body fluids and exosomes. For example, microarray-based expression analysis of five bone marrow (BM) samples from AML patients revealed high levels of circ-ANAPC7 (16). Yi et al. examined the expression of circ-VIM (circRNA derived from Vitementin) in bone marrow mononuclear cells (BMNCs) derived from the bone marrow of 113 AML patients and 42 healthy donors and observed upregulation of circ-VIM in AML patients (17). Moreover, they found that overexpression of circ-VIM was an independent poor prognostic factor and was markedly associated with shorter overall survival in AML patients. Ping et al. recently profiled the expression of circRNAs in the bone marrow of three AML patients and three controls (iron deficiency anemia) via circRNA-microarray analysis and documented elevated expression of circ_0009910 (hsa_circRNA_100053, circBase). circ_009910-miR-20a-5p upregulation was found in

Abbreviations: AML, Acute myeloid leukemia; circRNA, Circular RNA; miRNA, Micro RNA; CeRNA, Competing endogenousRNA; f-circRNA, Fusion circRNA; EIcircRNA, Exonic-intronic circRNA; CiRNA, Circular intronic RNA; EcircRNA, Exonic circRNA; TLR, Toll-like receptor; PML-RARα, Promyelocytic leukemia and the retinoic acid receptor α (RARα); IRES, Internal ribosome entry site; HIPK, Hypoxia-inducible protein kinase; XIAP, X-linked inhibitor of apoptosis protein; KEGG and GO, Kyoto encyclopedia of gene and genome and gene ontology; CLIP-seq, Crosslinking immunoprecipitation sequencing; qRT-PCR, Quantitative reverse transcriptase polymerase chain reaction; SiRNA, Small interfering RNA; CDK2, Cyclin-dependent kinase; AGO, Argonaute; QKI, Quanking I; EIF4A3, Elongation Initiation Factor; U1 snRNP, U1 small nuclear ribonucleoprotein; MRE, miRNA recognition element CRISPR-Cas9, Clustered regularly spaced palindromic repeats and CRISPR-associated gene/protein 9; NGS: Next-Generation Sequencing.

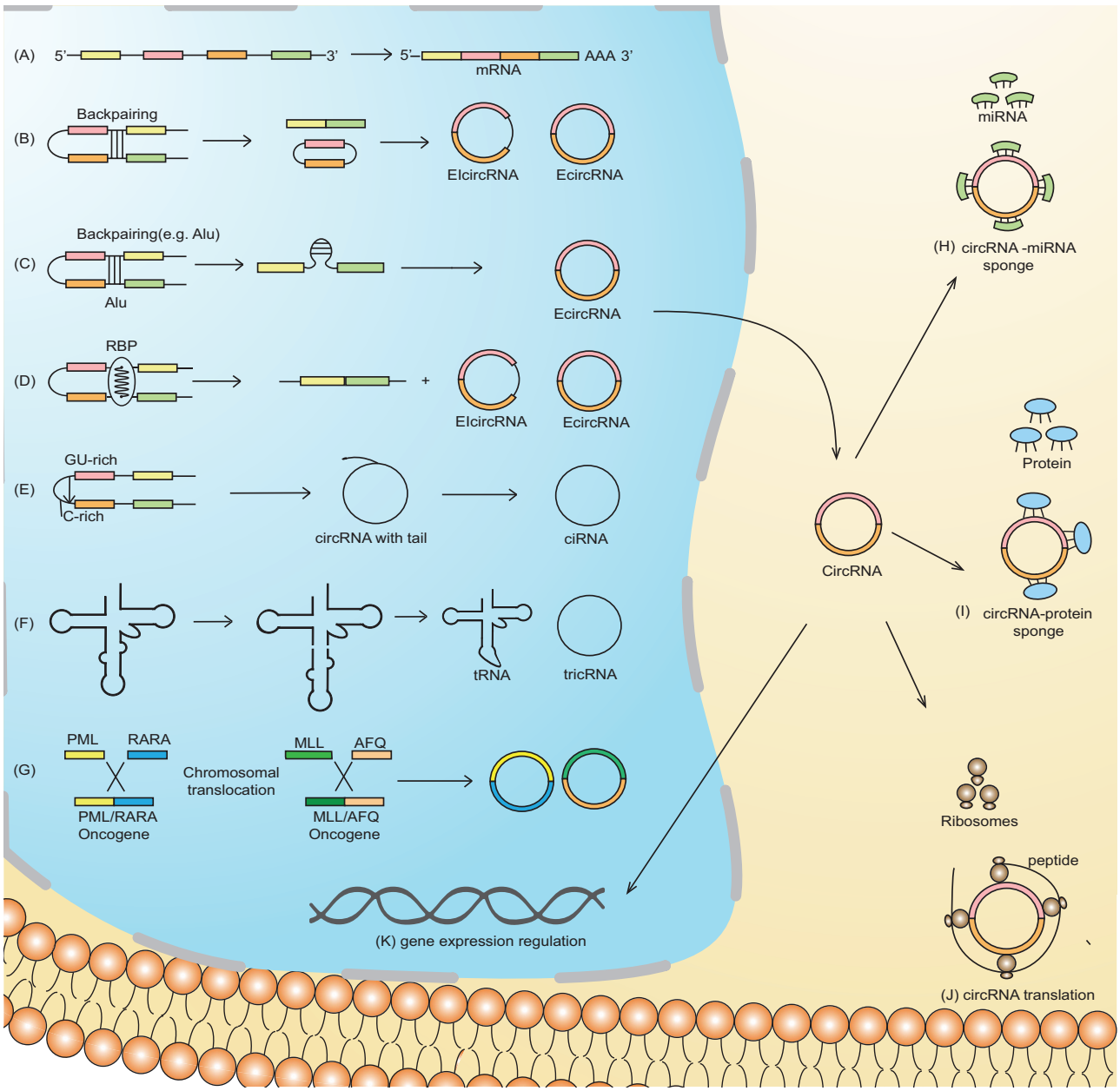

FIGURE 1 | Schematic representation of circRNA biogenesis. **(A)** Canonical pre-mRNA producing a mature mRNA. **(B)** Lariat-driven circularization, also called exon-skipping, is a process in which the partial folding of pre-mRNA allows the covalent binding of the exonic 5′-splice donor site with the 3′-end acceptor site, thus resulting in the skipping of exons. **(C)** Intron-pairing-driven circularization is characterized by base-pairing between different repetitive elements such as Alu repeats across different introns. The intronic sequence is subsequently cleaved to produce exonic circRNA (ecircRNA). **(D)** RNA-binding proteins (RBP) working as *trans*-acting factors vitally regulate the production of circRNA. Quaking (QKI) and Muscleblind (MBL/MBNL1) proteins preferentially bind to a particular sequence motif on a linear RNA, thus bridging the two bracketing introns together and circularizing the linear RNA to generate circRNA. **(E)** A novel circRNA known as ciRNA is dependent on a 7-nt GU-rich motif and an 11-nt C-rich sequence near the 5′-splice site and branchpoint site. The lariat structure containing the excised intron and exons is trimmed by the spliceosome followed by the degradation of the 3′-end tail, ultimately generating ciRNA. **(F)** tRNA intronic circular RNAs (tricRNAs) are produced as a product of the splicing of tRNA independent of pre-mRNAs, which is catalyzed by an enzyme in a presence of conserved tRNA sequence motif. **(G)** Chromosomal translocation results in the fusion of two genes producing fusion circRNA (f-circRNA). Functions of circRNA: **(H)** circRNAs may act as miRNA sponges by competing for miRNA binding sites, diminishing the effect of miRNA-mediated regulatory activities. **(I)** cricRNA may acts as protein (RNA binding protein), which regulate gene expression and are thus involved in various biological processes **(J)** circRNAs can be translated to form functional proteins, e.g., ribosome-associated circRNAs (ribo-circRNAs) harbor start codon and evolutionarily conserved stop codon encoding at least in protein domain circRNAs. **(K) c**ircRNAs (e.g., EIciRNAs and ciRNAs) may interact with transcription complexes and enhance the expression of their parental genes. Moreover, some of the nuclear enriched circRNAs may bind with the genomic DNA, forming an RNA-DNA triplex that can regulate the DNA replication process.

TABLE 1 | List of circular RNAs and their association with their respective miRNAs reported in AML.

Name of circRNA	Circ base ID	Expression regulation	miRNA target sponge	Functions	Host gene	Target gene(s)	References
Circ-ANAPC7	hsa_circRNA_101141	Up	miR-181 family	Oncogene, biomarker	ANAPC7	Numerous	(16)
circ-DLEU2	hsa_circ_0000488	Up	miR-496	Biomarker, Therapeutic target	DLEU2	PRKACB	(32)
–	hsa_circ_0004277-	Down	miR-138-5p miR-30c-1-3p miR-892b	Biomarker, Therapeutic target	WDR37 family	SH3GL2 PPARGC1A	(19)
circ-PAN3	hsa_circ_0100181	Up	miR-153-5p miR-183-5p	Drug resistance	PAN3	XIAP	(23)
circNPM1	hsa_circ_0075001	Up	miR-181	Biomarker	NPM1	TLR signaling pathway genes	(34)
–	hsa_circ_100290	Up	miR-203	Oncogene	SLC30A7	Rab10	(35)
circ_0009910	hsa_circRNA_100053	Up	miR-20a-5p	Biomarker	MFN2	RUNX3Rab27B Smad4	(18)

AML bone marrow patients compared to the control, and this upregulation predicted a poor outcome for AML patients. Further, the silencing of this circRNA inhibited the proliferation of AML cells and initiated apoptosis, highlighting the diagnostic and therapeutic potential of circ_009910-miR-20a-5p AML (18). Hsa_circ_0004277 (present on chromosome 10: 1125950-1126416) is encoded by WDR37, and the WDR37 family is involved in cellular events such as the cell cycle, apoptosis, and signaling pathways (3). Hsa_circ_0004277 was found to have reduced expression in both newly diagnosed untreated and relapsed AML patients (19). Interestingly, expression of hsa_circ_0004277 and WDR37 was found to be positively correlated during different AML stages, implicating WDR37 as a positive regulator of hsa_circ_0004277. Taken together, these reports suggest that circRNAs might be used as potential diagnostic and prognostic markers in AML.

circRNAs in Drug Resistance

One of the hallmarks of AML relapse following transient remission upon conventional treatment is the development of chemo-resistance to therapy, resulting in insensitivity to further treatment (20, 21). Multiple genes and non-coding sequences are involved in the development of drug resistance in AML (22). Various non-coding RNAs are emerging as important players in mediating drug resistance, and their targeting offers avenues for the development of novel treatment options. Nonetheless, studies on the potential involvement of deregulated circRNAs in AML drug resistance are just emerging. For example, circRNA-microarray expression analysis of doxorubicin (ADM)-resistant THP1 cells revealed a set of 49 circRNAs with altered expression, and elevated circPAN3 (product PAN3 gene) in recurrent and refractory AML was defined as a key player in drug resistance in AML (23). Of interest, circPAN3 is predicted to interact with **ten** miRNAs, miR-153-5p, miR-183-5p, miR-338-3p, miR-346, miR-545-3p, miR-574-5p, miR-599, miR-653-5p, miR-766-3p, and miR-767-3p. Functional analysis via the KEGG and GO databases predicted that the target genes of these miRNAs are associated with various cellular processes and signaling pathways involved in cancer (24–27). Moreover, miR-153-5p and miR-183-5p were found to interact with X-linked inhibitor of apoptosis protein (XIAP), which has been demonstrated as a drug resistance gene in AML (28, 29). Consistent with the predicted circPAN3-miRNA interactions, inhibiting circPAN3 expression reduces the expression of XIAP, and this effect was reversed by miR-153-3p- or miR-183-5p-specific inhibitors. In view of circPAN3 interaction with miR-153-5p and miR-183-5p, which in turn bind with XIAP, it can be predicted that circPAN3 may contribute to doxorubicin resistance in THP1 cells (23).

Similarly, overexpression of circPVT1 (derived from Plasmacytoma Variant Translocation 1 or PVT1) has also been associated with resistance to vincristine in AML (30), and knockdown of f-circM9 (a fusion circRNA) showed increased sensitivity to anti-leukemic drugs (31). These findings suggest that circRNAs can potentially be manipulated to reverse drug resistance (**Figure 2A**).

circRNAs in Regulating Cell Proliferation and Apoptosis

Based on the circRNA microarray expression dataset GSE94591, it was previously shown that circ-DLEU2 (hsa_circ_0000488) was highly expressed in AML samples (37). circ-DLEU2 is the splicing product of the DLEU2 gene, which is located on chromosomes (32). Further studies demonstrated that circ-DLEU2 negatively regulated miRNA-496, whose downstream target gene is PRKACB (**Figure 2B**). The protein encoded by PRKACB is the catalytic subunit of cyclic AMP-dependent protein kinase, which regulates various signaling processes such as proliferation and differentiation through cAMP. The expression of PRKACB was negatively regulated by miRNA-496, while circ-DLEU2 promoted the expression of PRKACB by sponging miRNA-496 (32). Consequently, increased expression of circ-DLEU2 facilitated the *in vitro* proliferation of leukemic cells, blocked apoptosis, and promoted the formation of AML tumor *in vivo*. On the other hand, Morens et al. reported the deletion of the DLEU2 locus and embedded miRNA cluster miR-15a/16-1 in adult leukemia but its transcriptional repression due

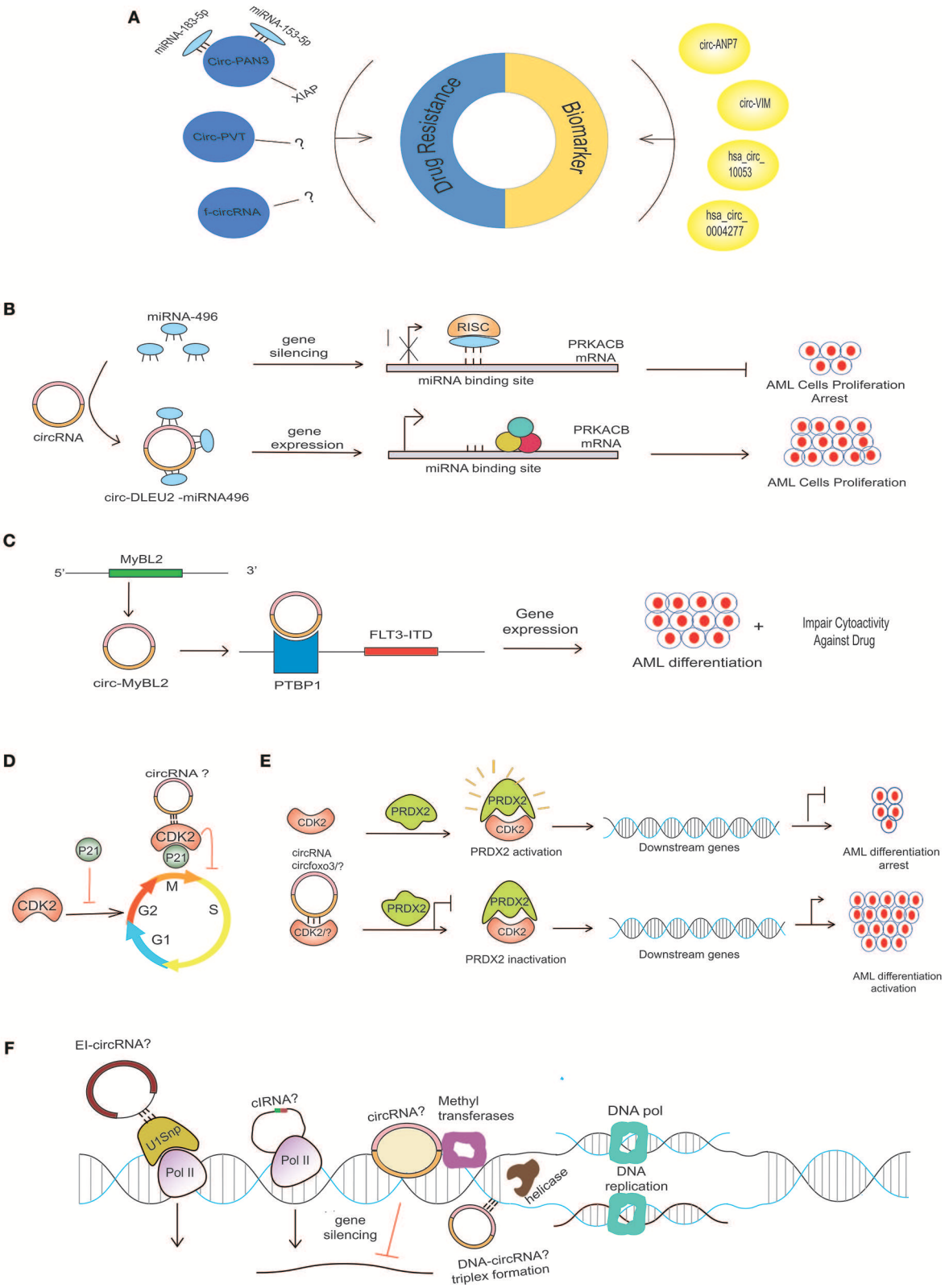

FIGURE 2 | Possible mechanism of circRNA in AML pathogenesis. **(A)** Aberrant expression of various circRNAs detected in BM samples of AML patients can be implicated in the diagnosis and prognosis of AML. circRNAs use different mechanisms to contribute to drug resistance in AML. CircPAN3 binds with two different

(Continued)

FIGURE 2 | miRNAs (miRNA-183-5p and miRNA-153-5p) to increase the expression of XIAP, which is demonstrated to be a drug-resistance gene in AML. Several circRNAs generated from fused genes, known as fusion circRNA (f-circRNA), can also contribute to drug resistance in an unknown mechanism. CircPVT and f-circRNA (a product MLL/AF9) also potentiates drug resistance in an unknown mechanism. **(B)** miRNA-496 bind to their respective sites on mRNA, causing PRKACB repression, but circDLEU2-miRNA-496 sponge formation allows the binding of translational machinery to the mRNA, thus causing gene derepression. The increased expression of PRKACB is associated with the proliferation of AML cells. **(C)** CircMYBL2 produced from the MYBL2 gene positively regulates the expression of the FLT3-ITD mutant gene by recruiting PTBP1 to the mRNA of the FLT3-ITD gene. **(D)** CDK2, a cyclin-dependent kinase enzyme regulating the cell cycle, is negatively regulated by P21. Circ-Foxo3 binds to CDK2 inhibitor 21 (p21), forming circfoxo3-p21-CDK2 complexes, thus preventing cell cycle progression. **(E)** Interaction of PRDX2 with CDK2 causes PRDX2 activation that, in turn, regulates the downstream genes, thereby arresting AML cell differentiation; however, the depletion of CKD2 due to its possible binding with circRNA? or degradation could diminish its interaction with PRDX2, thus rendering PRDX2 to its inactivated stage. The inactivation of PRDX2 is associated with AML differentiation activation (36). **(F)** Possible circRNAs with various gene expression-regulating abilities in AML. CircRNAsX (e.g., ElciRNAs and ciRNAs) may interact with transcription complexes and enhance the expression of their parental genes; other circRNA (EcircRNA) may repress the gene expression. Certain circRNA, like the lncRNA, may interact with the DNA by forming a circRNA-DNA triplex to regulate DNA replication.

to hypermethylation in young AML patients (38). The DLEU2 gene contains the locus for miR-15a/16-1, the expression of which is negatively regulated by the binding of BSAP (a transcription factor) to the DLEU2 promoter. Increasing the expression of miR-15a/16-1 by inhibiting BASP facilitates apoptosis and leukemic cell cycle arrest (39). These findings suggest that circ-DLEU2 is crucial for *in vitro* proliferation and tumor formation in AML and, although the DLEU2 gene is identified as a tumor-suppressor gene, it can also be implicated in leukemogenesis.

Recently, Fan et al. reported that hsa_circ_100290, upregulated in AML, possesses oncogenic potential by enhancing cellular proliferation, inhibiting cell-cycle arrest, and suppressing cell apoptosis (35). They found that hsa_circ_100290 binds to miR-293, the expression of which is downregulated not only in AML but also in other cancers (40, 41). In many cancer types, miR-293 has been found to suppress the proliferation, invasion, and migration of cancer cells. Moreover, the target protein of miR-293 is Rab10, a member of the RAS superfamily of small GTPases (42). Furthermore, they found that the expression of miR-293 was decreased while Rab10 was elevated in AML patients, and hsa-circ_100290 expression was negatively and positively correlated with miR-293 and Rab10, respectively.

PVT1, on 8q24, is a non-protein coding region that produces numerous ncRNAs (43). The mechanistic role of the PVT1 region and its non-coding transcripts in cancers remains elusive. Apart from the role of PVT1 lncRNA in stabilizing MYC (44), several groups have reported the role of PVT1 as an oncogene in AML (45). Moreover, the circRNAs generated from exon 2 of the PVT1 region, termed circPVT1, possess oncogenic roles in AML by enhancing proliferation. Knocking down f-circRNAs9 (a product MLL/AF9) resulted in apoptosis, while its expression inhibited the drug-induced apoptosis of leukemic cells both *in vitro* and *in vivo* (31). To further corroborate these results, K562 cells were transduced with f-circRNA9 and treated with the standard drugs cytarabine (Arac-C) and ATO. Cells expressing f-circRNA9 inhibited the apoptosis induced by ATO and Arac-C (compared to empty vector-transfected cells) (31). These findings support the potential value of therapeutically targeting oncogenic circRNAs in AML (**Figure 2B**).

Fusion-circRNAs in Leukemogenic Properties

Leukemia is characterized by prevalent chromosomal translocation events (46). These chromosomal rearrangements often result in the formation of fusion genes, which may produce not only oncogenic chimeric proteins (47) but also circRNAs known as fusion circRNA (f-circRNA) (**Figure 1G**) (48). The fusion chimeric gene MLL/AF9 yields two fusion circRNA products, f-circM9_1 and f-circM9_2, which promote leukemogenesis by promoting cell proliferation (31, 49). Hypoxia-inducible protein kinase (HIPK)-2 is a protein that stabilizes PML oligomerization, a process that is important in nuclear body formation. The disruption of nuclear bodies by the fusion protein of PML and the retinoic acid receptor α (RARα) (PML-RARα) is often associated with APL and is thus implicated in leukemogenesis (50). In another study, the mutation in HIPK2 showed its impaired localization with AML1 transcription factor complex. This complex serves as a target for leukemia-associated chromosomal translocations. The mutant HIPK2 negatively regulated AML1- and P53-dependent transcription and thus possibly contributes to the pathogenesis of leukemia (51). PCR amplification and sequencing of the site flanking the PML-RARα junction revealed that the paradigmatic fusion gene PML-RARα also produced multiple isoforms of f-circRNA with oncogenic potential (31). In this scenario, the circ-HIPK2 produced from the host gene might serve as a transcription co-activator in the formation of nuclear bodies. Moreover, investigating the circular RNA transcript of the fusion PML-RARα in the development of AML would constitute interesting future research.

Nucleophosmin 1 (NPM1) encodes a multifunctional, highly conserved nuclear chaperone protein that is involved in ribosomal biogenesis, apoptosis, and cell proliferation (52). An insertion into the exon12 of NPM1 produces an abnormal protein that abnormally localizes in the cytoplasm and contributes to leukemia formation in 35% of AML cases (37). A study showed the differential expression of several linear isoforms of the NPM1 gene in a cohort of AML patients (53). Moreover, the dislocation and deletion and high expression of NPM1 also contribute to the AML phenotype (48, 54). Recently, RNA-seq analysis identified circNPM1 variant hsa_circ_0075001 as a signature for defining patient subgroups. Comparative gene expression profiling of high vs. low hsa_circ_0075001 revealed several genes with differential expression. Gene expression pathway analysis of low and high circNPM1 expression groups revealed a significant difference in the TLR gene expression groups, particularly TLR4, TLR5, TLR7, and TLR8 (34).

TABLE 2 | Most commonly used databases containing circRNA-related information.

Name	Website	Features	References
circBase	http://www.circbase.org/	A merged and unified dataset containing sequence information of circRNAs	(55)
CircNet	http://circnet.mbc.nctu.edu.tw/	Tissue-specific circRNA expression profiles, circRNA-miRNA gene regulatory networks. Data is derived from transcriptomic sequencing studies	(56)
CircInteractome	https://circinteractome.nia.nih.gov/	Interaction of circRNA with miRNA and protein (RBP), primer and siRNA design	(57)
Circ2Traits	http://gyanxetbeta.com/circdb/	Interaction of circRNA with miRNA and long non-coding RNAs associated with disease or any other trait.	(58)
starBase	http://starbase.sysu.edu.cn/	Identify putative circRNA-miRNA and circRNA-RBP interaction networks	(59)
deepBase	http://deepbase.sysu.edu.cn/	Enable the annotation of sRNA, lncRNA, and circRNA.	(60)
circRNADb	http://reprod.njmu.edu.cn/circrnadb/circRNADb.php	A cancer-specific database. Enables the annotation of circRNA with protein-coding ability.	(36)
CIRCpedia	http://picb.ac.cn/rnomics/circpedia	Aims to annotate alternative back-splicing and alternative splicing in circRNAs	(61)
CIRCexplorer	https://circexplorer2.readthedocs.io/en/latest//	Aims to annotate alternative back-splicing and alternative splicing in circRNAs	(61)
CSCD	gb.whu.edu.cn/CSCD	Expression profiling of circRNAs obtained from RNA-seq data in cancer and normal tissues	(62)

WEB-BASED TOOLS FOR circRNA RESEARCH AVENUES

Because circRNA has multiple functions in various cancers, circRNA research is regarded as a hotspot in modern research. Various databases have been developed by researchers, mostly utilizing the data obtained from next-generation sequencing technology to shed light on the regulatory network and functional prediction of circRNA in various biological processes. The features of the most applied web-based tools are summarized in **Table 2**. The characteristics of the commonly employed databases are as follows. ***circBase,*** developed by Glazar et al., collects and integrates data related to circRNAs from public references and hosts information from various species such as model organisms. The database not only provides free access to the user to browse and download gene annotations but also allows the user to obtain evidence supporting the expression details of a particular circRNA (55). ***CircNet*** is a freely accessible database developed by Liu et al., which incorporates the transcriptomic datasets of novel and previously published circRNAs. The database provides tissue-specific circRNA expression profiling in terms of a heatmap. Furthermore, CircNet also depicts the regulatory network between circRNA, miRNA, and a particular gene (56). ***CircInteractome,*** developed by Dawood et al., is a freely accessible tool that enables the user to find the relevant miRNA- and RNA-binding protein sites on a particular circRNA. This database also facilitates circRNA-related research by allowing the researcher to design primers for qRT-PCR- and siRNA-based gene silencing. Moreover, CircInteractome can reveal the protein-coding abilities of a particular circRNA by deciphering its interaction with translational regulation factors and confirmation of IRES in circRNA (57). ***starBase*** depicts the interaction networks of circRNA with miRNA and RBP from 108 CLP-seq datasets generated by 37 different research studies. In addition, the other features of starBase v2.0 include miRNA-pseudogene interaction networks and miRNA-lncRNA interaction and competing endogenous RNA (ceRNA) functional networks (59). ***deepBase*** assembles NGS data from previous studies and enables users to annotate and discover small and long non-coding RNA and circRNAs. It also hosts features for decoding the evolution, expression profiles, and functions of non-coding RNAs across 19 species. Moreover, a spectrum of circRNA and lncRNA evolutionary patterns is also revealed by deepBase (60). Despite several worthwhile state-of-the-art toolkits for the identification, characterization, expression, and functional analysis of circRNA, problems such as a lack of a gold standard and overlap in predictions still need to be addressed. To this end, further innovation based on currently available methods with perfect precision and sensitivity should be pursued to overcome these challenges.

FUTURE PERSPECTIVE

Mechanism Exploration

One of the major functions of circRNA is sponging miRNA, thus regulating gene expression at an mRNA level. The dysregulation of miRNAs is implicated in the initiation and progression of leukemia (63, 64). miRNA sponging by circRNA can either transform into a leukemogenic property or its inhibition depending upon the type of miRNA inhibited by the circRNA. Numerous miRNAs with oncogenic potential have been reported in hematopoietic malignancies, e.g., miR22 causes the repression

of the TET2 gene, thus causing defective differentiation of leukemic cells (65). We expect future research to explore circRNAs with binding ability to oncogenic miRNA(s). Such research will be challenging, since multiple miRNAs can regulate a particular gene. For instance, an extensive network of TET2-targeting miRNAs (≥30 miRNAs) has been documented to inhibit the expression of the TET2 gene (66). These findings pave the way for the possible development of therapeutic strategies based on circRNA targeting mechanisms to rescue tumor suppressor gene expression.

circRNA-RBP Sponge

Mounting evidence is revealing the interaction of circRNA with RNA binding proteins (RBPs) and its potential functional aspects (67). These RBPs include Argonaute (AGO) (4, 68), RNA polymerase (RNAPII) (5), Muscleblind protein (MBL) (69), Quanking I (QKI) (10), and Elongation Initiation Factor (EIF4A3) (57). These RBPs regulate gene expression and are thus involved in vital cellular processes. CRISPR-Cas9-based screening of RBP in AML has revealed a network of physically upregulated interacting RBPs that are crucial in regulating the splicing of RNA and maintaining the leukemic condition. The depletion of RBM39 (as the main regulator of this network) results in altered splicing of the mRNAs essential for AML, thereby causing the mortality of AML cells (70). Recently, the interaction of hsa_circ_0004870 with RBM39 has been shown to be associated with enzalutamide resistance in castration-resistant prostate cancer (71). This finding highlights the importance of investigating circRNA interactions with RBM in AML. In addition, mutational profiling of leukemic patients has uncovered somatic genetic mutations in RBP that are associated with splicing, as extensively reviewed by (72). Very recently, the high expression circMYBL2, a product of the MYBL2 gene, was reported in AML patients within internal tandem duplication (ITD) mutations in the FLT3 (FLT3-ITD) gene. A positive regulation was observed between circMYBL2 and FLT3-ITD mutant kinase. In terms of mechanism, circMYBL2 was found to enhance FLT3-ITD mutant protein expression by facilitating the binding of PTBP1 to the mRNA of mutant FLT3 kinase. The knockdown of circMYBL2 not only inhibits the proliferation and supports the differentiation of AML cells both *in vitro* and *in vivo* but also compromises the cytoactivity of cells with the FLT3-ITD mutation against quizartinib (**Figure 2C**) (73). Circ-Foxo3 binds to cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 21 (p21), forming the circfoxo3-p21-CDK2 complex, thus preventing the cell cycle progression by inhibiting CDK2 (74) (**Figure 2D**). In AML, CDK2 blocks myeloid differentiation (**Figure 2E**). The ubiquitin (KLHL6-E3 ubiquitin ligase)-mediated proteasome degradation of CDK2 is associated with the activation of AML cell differentiation (75). The deletion of FOXO-1/3/4 family members facilitates the maturation of myeloid cells, AML cell death, and a diminution of leukemia-initiating cell function, thus improving animal survival (76). In the scenario of circ-FOXO3-p21-CDK2 ternary complex formation, it will be worthwhile to investigate the affinity of circ-FOXO3 or any other relevant circRNAs with CDK2 or any key cell cycle-regulating proteins, thus ultimately controlling the differentiation of cells in AML (**Figure 2D**). Molecular insights into understanding how malignancies are produced due to perturbation in pathways downstream of these sites will help in the identification of drug target regions.

Generally, owing to their stable structure, circRNAs are present in the cytoplasm, but some circular isoforms (EIcircRNA) are also present in the nucleus. These circular isoforms interact with chromatin modifiers, thereby causing repression or activation of the gene (77, 78). EIcircRNAs such as circEIF3J and circPAIP2 are associated with RNA polymerase II and recruit U1 small nuclear ribonucleoprotein (snRNP), which promotes the transcription of genes (8) (**Figure 2F**). In addition, certain circRNAs positively regulate the expression of their parent gene, such as in the case of the down-regulation of the ciRNA known as ci-ankrd52, which reduces the expression of ankrd52 without affecting the surrounding genes (**Figure 2F**) (5). circRNA derived from the SEP3 gene regulated the expression of the linear transcript by binding to its cognate DNA. The linear counterpart of circRNA-SEP3 with the same sequence binds to the DNA with weak affinity. Thus, circRNA-DNA formation presumably results in transcriptional repression and the generation of a SEP3 linear transcript with exon skipping (79). In addition, promoter-associated RNA (pRNA) suppresses the expression of the rRNA genes by recruiting DNMT3b to the target site of TTF-I (a transcription factor) via complementarity with the rDNA promoter (**Figure 2F**) (80). Watanabe et al. found that long non-coding RNA near the MYC gene could facilitate the amplification of MYC by influencing the movement of the replication fork (**Figure 2E**) (81). Another lncRNA known as ANRASSF1, produced from the opposite strand of a tumor-suppressor gene RASSF1 gene, binds with the promoter region of the parental gene, thus forming an RNA/DNA hybrid, and recruits polycomb repressive complex 2 protein (PRC2) to regulate its expression (82, 83). Like other ncRNAs, the circRNA may possibly affect DNA replication by binding with the genomic DNA, thus forming a DNA-RNA triplex (80). These findings highlight that circular and linear non-coding RNA may have the same binding pattern with the DNA and manifest unique effects on gene expression and DNA replication.

ANTI-ONCOGENIC POTENTIAL OF circRNA

Some circRNAs, for instance, circ-ZEB1.19, circZEB-1.17, circZEB1.5, and circZEB1.33, by trapping miRNA-200, are involved in the suppression of lung cancer progression (56). The downregulation of circular RNA produced from mitochondrial translocation optimization 1 homolog (MTO1), known as circMTO1, was reported in hepatocellular carcinoma tissue. circMOT1 decoys miR-9 to allow the expression of tumor suppressor gene p21 (84). The transfection of synthesized small circular single-stranded DNA-9 (CSSD-9), which mimics the circRNA, resulted in increased expression of various tumor suppressor genes such as LF17, CDH1, and LASS2 by absorbing

miR-9. The artificially synthesized CSSD-9, containing multiple binding sites for miR-9, impaired lung tumor progression and metastasis (85).

circRNAs were initially considered non-coding RNA due to their disability to recruit ribosome (9, 86). However, recent studies have documented the presence of an open reading frame (ORF) driven by the ribosomal machinery (13, 14). The circular transcript of the SNF2 histone linker PHD RING helicase (SHPRH) gene encodes a fully functional protein, 17kDa, termed circ-SHPRH, which suppresses the tumor transformation of glioma cells. In terms of mechanism, the circ-SHPRH protects SHPRH-146aa from ubiquitin-mediated proteasomal degradation, and subsequently, SHPRH ubiquitinates the proliferating cell nuclear antigen (PCNA) as an E3 ligase, thereby inhibiting cell proliferation (87). circRNA expression profiling of AML cell lines has identified that circKHLH8 is associated with clinical outcome, as its overexpression enhanced overall survival, with a higher level of platelets and reduced blast percentage in peripheral blood. Moreover, the inhibition of circFBXW7 facilitated the proliferation of AML cells, indicating the tumor-suppressive function of circFBXW7 in AML (88). Based on these findings, it is speculated that circRNA can work as an anti-oncogenic to promote the expression of tumor-suppressor genes by the derepression of miRNAs or by recruiting the transcription factor to the promoter of tumor suppressor genes.

TARGETED THERAPY

Of note, the circRNA-miRNA-mRNA axis is a widely accepted mechanism in the establishment of leukemia. Circularization-based approaches can be used as a treatment strategy. Generally, a circRNA contains multiple miRNA binding sites (MRE). Therefore, targeting the inhibition of circRNA expression rather than a single miRNA/gene offers treatment advantages. Inhibiting circRNA expression will promote the protective effect of the corresponding miRNA in suppressing oncogenes, for instance, XIAP (23), Rab10 (35), PRACKB (32), and TLR pathway-related genes (34). CRISPR-Cas9-based engineering is a robust tool for performing functional studies of circRNA in cancer (89). However, depleting the circRNAs without affecting the existing genes is a challenge. In order to achieve circRNA-specific knockdown and avoid the targeting of circRNA from its linear RNA, the guide sequence must be designed to target the back-splice junction (BSJ) site that is uniquely present in circRNA to avoid its binding with the linear transcript. The non-protein-coding property of circRNA makes it a tough choice for depletion by incorporating insertions/deletions (INDELs). A CRISPR-Cas-assisted homologous recombination approach may overcome the first challenge by replacing the circRNA gene with a marker gene (90). Moreover, strategies to avoid the off-target effects should be considered to circumvent undesirable mutations (91).

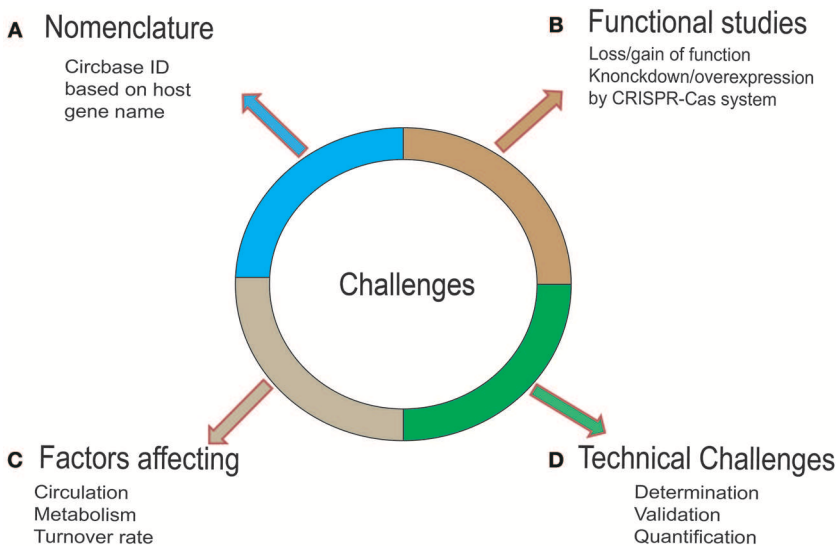

FIGURE 3 | Challenges and strategies for circRNA. **(A)** To date, circRNAs have been named based on various parameters by various available databases, creating chaos in scientific research. Therefore, a standardized and uniform naming system is needed. **(B)** To gain mechanistic insights into the biological functions of circRNA, mechanism exploration is needed. To this end, an optimized CRISPR-Cas system would offer a robust and easy platform to carry out these studies. **(C)** The knowledge of the fate of circRNA in a cell is not fully known. Owing to the high stability of these molecules, the accumulation of circRNAs may exert toxic effects on the cell. Studies have shown that the surplus circRNAs are exported out of the cells in vesicles such as exosomes. Further molecular studies are needed to uncover the factors controlling the circulation, metabolism, and turnover of the circRNA. **(D)** Factors such as the peculiarity of the back-splice junction in circRNA, sequence similarity with the linear transcript, and low abundance compared to its linear counterpart make determination challenging. A multistep strategy for overcoming this challenge is promising. These steps include the discovery/identification of circRNA (by RNA-sequencing or microarray expression analysis), validation of circRNA by qRT-PCR, Sanger sequencing, Nothern Blotting, fluorescent *in-situ* hybridization (FISH), and the quantification of circRNA by targeted RT-qPCR assay.

Nevertheless, CRISPR-Cas9-mediated deletion of the cdr1 locus has been achieved by Piwecka et al. (92). Similarly, disruption of the formation of RNA pairing reduces the expression of circRNA, and, under certain conditions, the knock-out of a circRNA has been achieved, for example, circGCN1LI in human PA1 cells (93). In addition, the currently developed tool for RNA targeting, CRISPR-Cas13, represents a promising method for targeting a specific circRNA (94). Cas13, a principal enzyme of the type VI CRISPR-Cas system guided by a 20 to 30-nt-long CRISPR RNA (crRNA), could specifically bind to the BSJ site and thus, in principle, be able to distinguish between the linear transcript and circular RNA. CRISPR-Cas-based circRNA engineering approaches can be harnessed for high-throughput screening (89), and future research using well-characterized guide RNA (gRNA) libraries designed for circRNAs may facilitate global screening studies.

CHALLENGES

Advancements in Next-Generation Sequencing (NGS) technologies and the availability of user-friendly bioinformatics tools have facilitated circRNA biology-related research in cancer. These advancements have helped in the transformation of circRNA research from considering it a redundant splicing byproduct to exploring its possible potential targeted therapeutic application in AML. However, the field is in its infancy and faces enormous challenges (**Figure 3**) and raises questions that need comprehensive consideration. First, the expense of NGS technologies limits the sample size, and there is a lack of gold-standard studies for data comparison. Second, despite extensive research related to the biogenesis of circRNA, knowledge on the metabolic processing of circRNA within cells is limited. Owing to the high stability of these molecules, the accumulation of circRNAs may have toxic effects on the cell. Studies have shown that surplus circRNAs are exported out of the cells in vesicles such as exosomes (95). Further molecular studies are needed to uncover the factors controlling the circulation, metabolism, and turnover of circRNA. Third, there is a lack of a gold-standard nomenclature system for circRNA. The naming of circRNA in future research should be based on the host gene along with the term "circ." Fourth, lack of perfection in the web-based tools, characterized by the low correspondence of the circRNAs tested by these different tools, is not helpful. To this end, in the future, we expect the development of a database integrating more comprehensive information about function and molecular mechanism. Finally, our knowledge of circRNA in AML is limited. In the last couple of years, researchers have shown that the circRNA expression pattern is related of clinical outcome and a poor prognosis. The most common mode of action of circRNA in AML is miRNA sponging, underscoring the need for investigation of other biological functions of circRNA such as the circRNA-RBP and circRNA-DNA regulatory networks. Although current research is being focused on the validation of these findings, further studies should focus on questions such as how circRNAs contribute to AML and the correlation between altered expression of circRNA and the mutational analysis of candidate genes that are crucial in alternative splicing events.

CONCLUSION

In conclusion, the role of circRNA in various cancers, including AML, is a major focus in cancer research. Although the detailed mechanisms of its biogenesis, transportation, function, and metabolism have not been fully studied, yet the glimpses provided by modern research suggest that circRNA as a potential candidate for AML diagnosis and therapy.

AUTHOR CONTRIBUTIONS

MJ, QZ, and LS designed and conceived the study. TS, BC, ZH, and TX helped with figure design and literature selection and drafted the manuscript. MF, LS, YW, and SP revised the language. QY helped in manuscript formatting and language revision. All of the authors read and approved the final manuscript.

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REVIEW
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Circular RNAs and RNA Splice Variants as Biomarkers for Prognosis and Therapeutic Response in the Liquid Biopsies of Lung Cancer Patients

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Lung cancer, including non-small cell lung carcinoma (NSCLC), is the most frequently diagnosed cancer. It is also the leading cause of cancer-related mortality worldwide because of its late diagnosis and its resistance to therapies. Therefore, the identification of biomarkers for early diagnosis, prognosis, and monitoring of therapeutic response is urgently needed. Liquid biopsies, especially blood, are considered as promising tools to detect and quantify circulating cancer biomarkers. Cell-free circulating tumor DNA has been extensively studied. Recently, the possibility to detect and quantify RNAs in tumor biopsies, notably circulating cell-free RNAs, has gained great attention. RNA alternative splicing contributes to the proteome diversity through the biogenesis of several mRNA splice variants from the same pre-mRNA. Circular RNA (circRNA) is a new class of RNAs resulting from pre-mRNA back splicing. Owing to the development of high-throughput transcriptomic analyses, numerous RNA splice variants and, more recently, circRNAs have been identified and found to be differentially expressed in tumor patients compared to healthy controls. The contribution of some of these RNA splice variants and circRNAs to tumor progression, dissemination, or drug response has been clearly demonstrated in preclinical models. In this review, we discuss the potential of circRNAs and mRNA splice variants as candidate biomarkers for the prognosis and the therapeutic response of NSCLC in liquid biopsies.

Keywords: cancer biomarkers, circular RNAs, liquid biopsies, lung cancer, RNA splice variants

INTRODUCTION

Lung cancer is the most common cause of cancer-related deaths (Ferlay et al., 2015; Siegel et al., 2017). Non-small cell lung cancer (NSCLC) comprises mainly lung adenocarcinoma, squamous cell lung carcinoma, and large cell lung carcinoma. It represents about 85% of total lung cancer patients. Over the past decades, the emergence of therapies [such as those targeting the epidermal growth factor receptor (EGFR), the anaplastic lymphoma kinase (ALK), or the immune checkpoints programmed cell death (PD-1)/programmed death ligand (PD-L1)] has led to an unprecedented

improvement of progression-free survival in a subset of advanced NSCLC patients. However, the long-term survival for NSCLC patients remains very poor and is still closely related to the stage of the disease (Chansky et al., 2017; Kris et al., 2017). This poor prognosis is mainly due to the intrinsic aggressiveness of NSCLC, the late diagnosis of the disease, and the recurrent development of resistance to targeted therapies. Therefore, the identification of biomarkers for early detection and selection of high-risk subjects as well as the characterization of predictive biomarkers (at both baseline and progression) of therapeutic response are urgently needed. This could improve the clinical management of these patients and help to understand the tumor progression mechanisms under treatment. In this context, less-invasive, specific, reproducible, and sensitive pre-analytical and analytical methods for the collection and analysis of tumor samples have to be developed, improved, and standardized.

Circular RNAs (circRNAs) are generated by back splicing. Unlike linear RNAs, they have closed-loop structures coming from the ligation of exons, introns, or both (Dragomir and Calin, 2018; Wang et al., 2019). Although first described in the early 1970s, circRNAs were until very recently considered as the by-products of splicing without any important biological functions. These recent years, the development of next-generation sequencing technologies together with the improvement of bioinformatic pipelines has allowed the identification of numerous circRNAs that are differentially expressed (either up- or down-regulated) in cancer patients compared to normal controls (Dragomir and Calin, 2018). Therefore, a new area of research is now emerging to investigate the role of circRNAs as candidate biomarkers for the diagnosis or prognosis of cancer.

In eukaryotes, gene expression is finely controlled by complex regulatory processes that affect all steps of RNA expression, extending from transcription to maturation in the nucleus, mRNA cytosolic export, stability, and translation. One of the crucial steps is the pre-mRNA constitutive splicing process, which removes intronic sequences from the pre-mRNA and joins exonic sequences to form the mature mRNA. Another level of regulation is represented by pre-mRNA alternative splicing (Stamm et al., 2005; Hallegger et al., 2010). Through the use of alternative promoters, alternative polyadenylation sites, intron retention, or alternatively spliced exons (exon cassette or mutually exclusive exon), this process generates several coding mRNA splice variants from the same gene. Therefore, one of the main consequences of alternative splicing is to remodel the proteome through the synthesis of various protein isoforms with different biological activities. This process is tightly controlled across different tissues and developmental stages, and the dysregulation of alternative splicing has been closely associated with various human diseases including cancer (Pan et al., 2008; Wang E.T. et al., 2008). Thus, a deepened characterization of pre-mRNA processing events that are deregulated in tumors is required to uncover new oncogenic mechanisms as well as to design alternative therapeutic strategies.

In this review, we discuss the importance of circRNAs and RNA splice variants as cancer biomarkers, with a focus on lung cancer. We also discuss the pros and cons of RNA liquid biopsies as promising but challenging approaches to identify potential RNA biomarkers in cancer.

RNA LIQUID BIOPSIES: PROMISING TOOLS FOR THE DETECTION OF CANCER BIOMARKERS?

In clinical practice, repeated tissue biopsies can be used to monitor tumor evolution. However, these are invasive procedures with complication risks for patients. In addition, single-site tissue biopsy represents a snapshot of the tumor, often provides insufficient materials, and is subjected to selection bias resulting from tumor heterogeneity. Tumor liquid biopsies can be considered as a non-invasive surrogate of tissue biopsies. Furthermore, circulating markers more likely reflect systemic tumor burden and depict intratumoral heterogeneity that occurs in metastatic lesions (Cai et al., 2015; Siravegna et al., 2017; Rolfo et al., 2018). Liquid biopsies are therefore a highly relevant alternative to tissue biopsies for initial molecular diagnosis and management of tumor progression, especially following treatment.

Beside proteins, various tumor-derived components can be isolated from blood including circulating cell-free tumor DNA (ctDNA), cell-free tumor RNA (ctRNA), circulating exosomes, tumor educated platelets (TEPs), and circulating tumor cells (CTCs) (Joosse and Pantel, 2015; Reclusa et al., 2017; Raez et al., 2018). It was previously found that tumor patients have higher levels of ctDNA compared to healthy donors, and metastatic patients even more than non-metastatic patients (Leon et al., 1977). In NSCLC patients, ctDNA sequencing of tumor plasma was reported to be as useful as tumor tissue genotyping to follow the appearance of specific mutations such as EGFR(T790M) mutations at the time of tumor progression (Oxnard et al., 2016). More recently, Abbosh et al. (2017) identified differential phylogenetic ctDNA profiles associated with adjuvant chemotherapy resistance and lung cancer relapse and metastasis. Therefore, detection of ctDNA in the plasma could also be used to track the appearance/selection of lung cancer subclones during progression upon treatment.

Circulating RNAs are also detected in tumor liquid biopsies. As an example, it has been shown that platelets sequester tumor-related RNAs by a microvesicle-dependent mechanism, resulting in the generation of TEPs (Best et al., 2015). Importantly, RNA sequencing of platelets from 60 patients with NSCLC (56 metastatic) and 55 healthy donors identified a specific tumor signature (Best et al., 2015). Plasma cell-free RNA has also been investigated for a long time (Kopreski et al., 1999) and used for the measurement of RNA transcripts of fusion genes (SLIT, ALK, ROS1, and RET) by reverse transcription polymerase chain reaction (RT-PCR) in lung cancer (Zhao et al., 2014). New gene fusions such as those leading to the constitutive activation of the tropomyosin receptor kinase TrkA encoded by the NTRK1 gene have been identified in lung cancer patients (Vaishnavi et al., 2013; Amatu et al., 2016). As the TrkA inhibitor larotrectinib was very recently approved by the FDA for patients with advanced solid tumors carrying NTRK gene fusions, the

detection of these transcripts in plasma could be a valuable tool for the management of these patients. Circulating RNAs have substantial advantages over ctDNA: (1) RNA expression level is highly dynamic and fluctuates according to the proliferative state or metabolism of cancer cells; (2) the expression pattern of RNA molecules, including splice variants or long non-coding RNAs (lncRNAs), is highly specific of tissue types or disease stage; and (3) focusing on RNA provides a combined analysis of a multitude of RNAs (lncRNAs, miRNAs, piwi-interacting RNAs, fusion transcripts, splice variants, or RNA modifications). These last years, owing to the development of high-throughput sequencing technologies, the number of RNAs belonging to each category has extensively increased, providing an unlimited reservoir of potential cancer biomarkers in various cancer types (Nigita et al., 2018; Obermannova et al., 2018). Conversely, circulating RNAs have substantial disadvantages over ctDNA: (1) circulating free RNA is not stable; (2) RNA transcription varies between people according to different variables such as gender, diet, and age; and (3) different factors such as time of blood draw, blood collection tube, blood fraction (i.e., platelet-rich versus platelet-free plasma), complete blood count, or RNA isolation protocols could influence RNA abundance levels in body fluids, both inside and outside extracellular vesicles. Therefore, carefully annotated health control libraries as well as well-standardized protocols for sample collection and analysis are needed before studying complex diseases such as cancer (Yuan et al., 2016).

CIRCRNAS: A PROMISING SOURCE OF BIOMARKERS IN LUNG CANCER?

CircRNAs: General Considerations

CircRNAs are generated from linear pre-messenger RNAs through a process termed "back splicing," in which the 3' and 5' ends are joined together, leading to a covalently uninterrupted loop. Although circRNAss were first discovered in the 1970s, they were considered for a long time as misspliced by-products without any important biological roles. In 2012, numerous circular transcripts were discovered and annotated in mammalian cells (Salzman et al., 2012). CircRNAs originate from several types of RNA molecules including exons, introns, long non-coding RNAs, antisense transcripts, or intergenic regions (Dragomir and Calin, 2018). In addition, circRNAs originating from coding regions can contain multiple exons and sometimes also include intronic regions (Maass et al., 2017). This could be dependent on the average length of the pre-mRNA exons and of the flanking introns. Some circRNAs such as Circ-ZNF609 are translated with a role in myogenesis in that case (Legnini et al., 2017). Interestingly, some circRNAs are more abundantly expressed than their associated linear mRNAs (Salzman et al., 2013; Maass et al., 2017). Thus, circRNA formation could result from distinct post-transcriptional regulation by competing with the formation of linear mRNA from the same pre-mRNA (Ashwal-Fluss et al., 2014).

Briefly, three main mechanisms lead to back splicing (Dragomir and Calin, 2018). The first mechanism is defined by exon skipping and intron lariat building, which brings distant exons together and induces the circularization of skipped exons. As a consequence, three different mRNA molecules are formed: an intron lariat, a circRNA, and a linear mRNA with skipped exons (Zaphiropoulos, 1997; Jeck et al., 2013; Kelly et al., 2015). The second mechanism comes from the circularization of exon/exon by the complementary pairing of the flanking introns (Dubin et al., 1995; Liang and Wilusz, 2014; Zhang et al., 2014). Introns that paired often contain inverted ALU repeats (Jeck et al., 2013). The third mechanism is controlled by RNA binding proteins (RBPs) such as quaking (QKI) and musclebind (MBL) proteins, which bind the neighboring introns of the future circular exons and dimerize, creating an RNA loop (Ashwal-Fluss et al., 2014; Conn et al., 2015).

The biological functions of circRNA are scarce (Hentze and Preiss, 2013). Several studies have shown that most of circRNAs contain some, although not multiple, miRNA response element (MRE) (Guo et al., 2014). As a consequence, the role of circRNAs as miR sponges has been extensively investigated and has been reported as an indirect mechanism to increase the expression of numerous mRNAs (Hansen et al., 2013; Memczak et al., 2013; Tay et al., 2014). The majority of circRNAs does not associate with ribosomes or the translational machinery (Guo et al., 2014). However, in some cases, the presence of an internal ribosome entry site (IRES) in the circRNA sequence would allow translation, at least *in vitro* (Chen and Sarnow, 1995). In agreement with these results, it was reported that both circZNF609 and circMBL associate with polysomes (at low levels) and are translated into short proteins, which contain a functional domain (Legnini et al., 2017; Pamudurti et al., 2017). The exact function of these peptides is yet unknown. Similar to miRNA sponging, circRNAs also bind RBPs such as MBL (Ashwal-Fluss et al., 2014), SR proteins (SRSF1), (Barbagallo et al., 2018), or IGF2BP3 (Schneider et al., 2016). Again, the functional consequences of these interactions remain elusive and controversial. Different explanations have been proposed: (1) circRNAs could be vehicles for RBPs, allowing their delivery in a specific subcellular localization; (2) circRNAs could be sponges for RBPs and could inhibit their function by sequestration; (3) circRNAs could act as a platform for multiple RBPs, allowing their interaction; and (4) circRNAs could bind RBPs and induce allosteric changes that regulate their functions.

Up to now, the signaling pathways that control the formation of circRNA loops remain largely unknown. Notably, the mechanisms that deregulate circRNA expression in cancers are unknown. In Drosophila, it was found that SR (serine rich) and hnRNP proteins, which are crucial regulators of both constitutive and alternative splicing, act in a combinatorial manner to regulate back splicing of many pre-mRNAs. This suggests that the spliceosome machinery activity controls the balance between linear and circular mRNAs (Kramer et al., 2015). Consistent with this idea, depletion or pharmacological inhibition of the spliceosome components of the SF3b or SF3a complexes, which include SR proteins or hnRNPs, increases the steady-state levels of circRNAss while concomitantly decreases the linear mRNAs (Liang et al., 2017). These results demonstrate

that inhibition or slow-down of canonical pre-mRNA processing events shifts the steady-state output of protein-coding genes toward circRNAss. Interestingly, mutations in spliceosome genes such as SF3B1, SRSF2, and U2AF1 are prevalent in several cancers, including lung cancers (Chabot and Shkreta, 2016). Therefore, it is tempting to speculate that these mutations as well as other molecular alterations (amplification, translocation. . .) of specific cancer-related genes may affect circRNA biogenesis and contribute to disease progression. Although this hypothesis has not been validated yet, a link between circRNA expression, KRAS mutation, and c-MYC overexpression has been reported in lung and colorectal cancers (Dou et al., 2016; Gou et al., 2017).

CircRNAs as Prognostic Biomarkers in Lung Cancer

Many studies have analyzed circRNA expression in lung cancer cell lines (Panda et al., 2017; Tian et al., 2017), as well as in tissue samples and liquid biopsies (plasma) from lung cancer patients (Hu et al., 2018; Ma Y. et al., 2018; Wang et al., 2019). Up to now and based on PubMed database, about 50 papers have identified circRNAs in lung cancer. As most of these papers were published last year, it is obvious that the number of studies will exponentially increase in the future. In 2018, Ding et al. (2018) performed a genome-wide transcriptome profiling of circRNA in paired lung adenocarcinoma and healthy lung tissues using ribosomal RNA-depleted RNA sequencing. They identified 9,340 circRNA candidates. Although circRNAs were often weakly expressed and identified on less than 10 reads for more than half of them, these results are consistent with their wide expression in lung adenocarcinoma. **Supplementary Tables 1, 2** list circRNAs that are either up-regulated (**Supplementary Table 1**) or down-regulated (**Supplementary Table 2**) in lung cancer compared to normal lung. Below are examples of these circRNAs. Some of them have been proposed as potential diagnostic and/or prognostic biomarkers. Mechanistically, most of these circRNAs act as miR sponges.

Many circRNAs are down-regulated and are thus considered as tumor suppressors. Down-regulation of the circRNA transcript of the tumor suppressor gene ITCH was reported in a large series of lung cancer patients (Wan et al., 2016). Mechanistically, circ-ITCH sponged miR-7 and mir-214. Its down-regulation induced the activation of the Wnt/beta-catenin signaling pathway through the up-regulation of T-cell factor, beta-catenin, c-Myc, and cyclin D1 (Wan et al., 2016). The down-regulation of various circRNAs targeting ZEB1 (circ-ZEB1.5, circ_ZEB1.19, circ_ZEB1.17, and circ_ZEB1.33) increased the level of miR-200, which promoted tumor lung metastasis (Liu et al., 2016; Wang F. et al., 2016). Expression of circPTK2 was reduced in NSCLC cells. This activated TGF-beta-induced epithelial to mesenchymal transition and invasion through a miR-429/miR-200b-3p axis and an increased expression of SNAIL (Wang L. et al., 2018). Decreased expression of circ-FOXO3 was also found in lung cancer (Zhang Y. et al., 2018). As circ-FOXO3 binds focal adhesion kinase (FAK) and hypoxia inducible factor 1 (HIF1) alpha proteins and prevents their nuclear translocation (Du et al., 2017), it was assumed that its down-regulation enhanced the angiogenic and invasive properties of FAK and HIF1. Finally, the low expression of Hsa_circ_0033155 (Gu et al., 2018) and hsa_circ_100395 (Chen et al., 2018) was associated with a poor prognosis in NSCLC patients.

Conversely, many circRNAs are up-regulated in lung cancer and are thus considered as tumor promoters. circ-404833 and circ-406483 are highly expressed in the tissues of early-stage lung cancer patients compared to control tissues (Zhao et al., 2017). Up-regulation of hsa_circ_0012673 was observed in lung adenocarcinoma tumor samples when compared to pair-matched adjacent non-tumoral tissues and was associated with tumor size (Wang X. et al., 2018). Moreover, expression level of numerous circRNA, such as hsa_circ_0013958 (Zhu et al., 2017), hsa_circ_0000064 (Liu et al., 2018; Zhang S. et al., 2018), ciRS-7 (Yan et al., 2018), circRNA_102231 (Zong et al., 2018), hsa_circRNA_103809 (Liu et al., 2018), circFADS2 (Zhao et al., 2018), circ_0067934 (Wang and Li, 2018), hsa_circ_0000729 (Li S. et al., 2018a), circ-BANP (Han et al., 2018), circ_0016760 (Li Y. et al., 2018), hsa_circ_0020123 (Qu et al., 2018), and circPRKCI (Qiu M. et al., 2018), was associated with TNM stage and lymphatic metastasis in lung cancer patients. These cirRNAs have been proposed as potential biomarkers for the early detection and screening of lung cancer. Mechanistically, *in vitro* studies also demonstrated that circRNAs promote cancer cell proliferation, migration, and invasion as well as prevent apoptosis by acting as miR sponges that regulate gene expression. As an example, hsa_circ_0013958 promoted cancer cell proliferation and invasion by acting as a miR-34 sponge to up-regulate cyclin D1 expression (Zhu et al., 2017). Circ-MAN2B2 promoted the expression of FOXK1 by sponging miR-1275, leading to cell proliferation and invasion (Ma X. et al., 2018). Expression of Hsa_circ_0007385 was associated with cell proliferation, invasion, and metastasis, which could be related with its sponge activity against miR-181 (Jiang et al., 2018). In A549 and H1299 lung adenocarcinoma cells, hsa_circ_0000064 promoted cell proliferation, migration, and invasion and prevented apoptosis (Luo et al., 2017). Some of its targets included cell cycle regulators ($p21^{WAF1}$, CDK6, and cyclin D1) and apoptotic factors (caspase-3, caspase-9, and bax). Hsa_circ_0012673, a known miR-22 sponge, enhanced the proliferation of lung adenocarcinoma cells through up-regulation of ErbB3 expression (Wang X. et al., 2018). As circRNA_100876 is a sponge for miR-136 that targets the matrix protease MMP13, increased MMP13 activity may explain the association between up-regulation of circRNA_100876 and lung tumor progression (Yao et al., 2017). Circ-FGFR3 activated a galectin-1/AKT/ERK signaling pathway by sponging miR-22-3p, and promoted cell invasion and proliferation (Qiu B.Q. et al., 2018). Very recently, Li L. et al. (2018) identified FECR1, an exonic circRNA resulting from the back splicing of exon 4-2-3 of the friend leukemia virus integration 1 (FLI1) transcript, as a driver oncogene that promoted tumor metastasis in small cell lung carcinoma (SCLC). They showed that FECR1 sponges miR584-3p and induces the activation of the rho-associated coiled-coil kinase 1 (ROCK1), which promotes metastasis. Interestingly, Qiu M. et al. (2018) found that amplicon 3q26.2, which is highly susceptible to gene mutation in lung

cancer, produces circPRKCI and promotes cell proliferation and migration through a circPRKCI-miR545/589-E2F7 axis.

As a whole, these studies clearly indicated that circRNAs contribute to the progression of lung cancer, especially NSCLC.

Circulars RNAs and Lung Cancer Response to Therapies

Although targeted therapies and immunotherapies have revolutionized the therapeutic management of NSCLC patients, resistance develops. Therefore, further research is needed to identify predictive biomarkers for the follow-up of patients. To our knowledge, only very few studies have investigated the regulation of circRNAs (biogenesis, localization, and functions) in response to treatment. Given the role of circRNAs in lung tumor progression, this is an important topic, although it remains challenging. Moreover, as the access to tumor biopsies before/after relapse remains difficult, the detection of circRNAs in liquid biopsies is a promising alternative for the management of post-treatment follow-up.

Zhou et al. (2019) recently showed that hsa_circ_0004015 increases the resistance of HCC827 NSCLC cell line to gefitinib, an EGFR-tyrosine kinase inhibitor currently used in clinic. Through its ability to sponge miR-1183, hsa_circ_0004015 induced the expression of the 3-phosphoinositide-dependent protein kinase 1 (PDPK) and stimulated AKT pathway (Zhou et al., 2019). Using a high-throughput circRNA microarray, Xu et al. (2018) found a significant up-regulation of 2,909 circRNAs and down-regulation of 8,372 circRNAs in taxol-resistant A549 lung adenocarcinoma cells compared to parental cells. Interestingly, they highlighted circRNA/miRNA networks, suggesting that circRNAs also acted through miRNAs sponging in this context. They found that the most significantly enriched pathways for aberrant circRNA-related host genes include integrin, EGFR, VEGFR, and rho GTPase signaling or degradation of the extracellular matrix, which are all involved in the progression of cancers following chemotherapeutic treatment. Altogether, these studies strongly suggested that dysregulated circRNA expression contributes to the resistance of NSCLC tumors to targeted therapies as well as to chemotherapies.

Another role of circRNAs during cancer growth is the formation of fusion circRNAs (f-circRNAs) produced by chromosomal translocations. These fusion circRNAs were first identified in leukemias (Guarnerio et al., 2016). The EML4-ALK fusion is the result of a chromosomal rearrangement between the N-terminal portion of EML4 and the tyrosine kinase domain of ALK. ALK rearrangements are more prevalent in younger NSCLC patients, females, and never or former light smokers (Shaw et al., 2011). Importantly, EML4-ALK-positive patients derive clinical benefits from ALK tyrosine kinase inhibitors such as crizotinib (Shaw et al., 2013). However, despite initial good responses, tumor progression is systematically observed due to the emergence of resistance mechanisms (Katayama et al., 2012). Interestingly, Rosell's group detected EML4-ALK rearrangements in RNA isolated from platelets (Nilsson et al., 2016). During the course of the disease, the presence of EML4-ALK fusion transcripts in platelets correlated with the clinical outcome in response to crizotinib treatment. The same group also demonstrated the presence of EML4-ALK fusion transcripts in the plasma by using RT-PCR. Moreover, a F-circRNA derived from back splicing of the EML4-ALK fusion gene (F-circEA-2a) was recently identified (Tan et al., 2018). F-circEA-2a promoted cell invasion and migration but not cell proliferation and colony formation in NSCLC (Tan et al., 2018). F-circEA-2a was detected in tumor tissues but not in the plasma of EML4-ALK-positive patients. It remains to be determined whether F-circEA-2a expression varies upon treatment with crizotinib and whether F-circEA-2a could represent a new predictive RNA biomarker of response to targeted therapies.

CircRNAs as Valuable Biomarkers in Liquid Biopsies: Pros and Cons

The features of a qualified biomarker include stability, sensitivity, specificity, accuracy, and reproducibility (Henry and Hayes, 2012). CircRNAs encompass some of these features. Due to the covalently closed-loop structure lacking free 5' and 3' ends, circRNA molecules are highly resistant to exonuclease RNAse R (Jeck et al., 2013) and are thus much more stable than linear RNAs (Memczak et al., 2013; Enuka et al., 2016). Hence, the average half-life of circRNAs in plasma exceeds 48 h, while that of mRNAs is 10 h on average (Jeck and Sharpless, 2014). In addition, circRNAs are tissue and developmental stage specific. They are differentially expressed between cancerous and non-cancerous tissues (Memczak et al., 2013). Their differential pattern of expression allows to distinguish cancer types and/or histological subtypes (Salzman et al., 2013). Therefore, owing to these general features, circRNAs have been considered as highly suitable biomarkers for liquid biopsies. circRNAs are detectable in body fluids such as saliva (Bahn et al., 2015) or whole blood (Memczak et al., 2015), and are also abundant in exosomes (Li et al., 2015). circRNAs are detected in the free-floating cells inside body fluids such as circulating blood cells (platelets and erythrocytes) and CTCs. Maass et al. (2017) found that platelets exhibit the highest expression of circRNAs among 20 different tissues tested. Although the expression of circRNAs is abundant in the plasma/serum, many of these circulating circRNAs do not discriminate between cancer and non-cancer patients (Henry and Hayes, 2012). Thus, only a few circRNAs could be differentially expressed in cancerous patients liquid biopsies. As an example, Li L. et al. (2018) recently detected FECR1 circRNA in exosomes purified from SCLC patient sera. The expression of serum-exo-FECR1 was aberrantly increased in SCLC patients compared with normal healthy donors and was associated with disease progression following chemotherapy (Li L. et al., 2018).

Therefore, before using circRNAs as cancer biomarkers in liquid biopsies, we have to carefully decipher the biological material that is the most relevant for detection (plasma, serum, whole blood, and platelets) and to better characterize circRNA expression level in relevant disease controls (healthy donors) and relevant control cells. We also have to optimize and standardize the pre-analytical methods of circRNA purification as well as the analytical procedures and bioinformatic pipelines.

RNA SPLICE VARIANTS AS BIOMARKERS OF CANCER PROGRESSION AND RESPONSE TO THERAPIES IN LUNG CANCER

Alternative Splicing and Cancer Progression

It is now well known that abnormal pre-mRNA alternative splicing, through either consensus splice sites mutations or deregulated expression and/or mutations of splicing factors, occurs in cancer and leads to the differential expression of numerous RNA splice variants (Pio and Montuenga, 2009; Syafrizayanti et al., 2018; **Figure 1**). In this setting, we previously reported the up-regulation of SRSF1 and SRSF2 proteins and SRPK1 and SRPK2 kinases in NSCLC and neuroendocrine lung tumors compared to normal lung tissues (Edmond et al., 2011; Gout et al., 2012). Moreover, and consistent with a role of aberrant splicing in lung tumors, alterations in the splicing pattern of several genes including CD44, Bcl-x, cyclin-D1, FHIT, KLF6, or VEGF-A were associated with lung cancer progression (Pio and Montuenga, 2009; Syafrizayanti et al., 2018; **Table 1**). Recently, we showed that $VEGF_{165}b$, a splice variant of VEGF-A, regulates a proliferative and invasive autocrine loop in lung adenocarcinoma cell lines and accumulates in lung adenocarcinoma patients, where it correlates with lymph node metastases (Boudria et al., 2018). Interestingly, we detected an increase in the $VEGF_{165}b$ protein in the serum of lung adenocarcinoma patients compared to healthy donors. Moreover, we demonstrated that sVEGFR1-i13, a splice variant of the vascular endothelial growth factor receptor 1 (VEGFR1), is up-regulated in squamous lung carcinoma and promotes the proliferation and survival of tumor cells *via* a beta1 integrin-dependent autocrine loop (Abou Faycal et al., 2018). Altogether, these results indicated that abnormal pre-mRNA splicing contributes to the progression of lung tumors.

As many studies demonstrate aberrant splicing in cancer, therapeutic strategies to correct these alterations have been developed. Below are some of the strategies that produced very encouraging results in preclinical models of different cancer

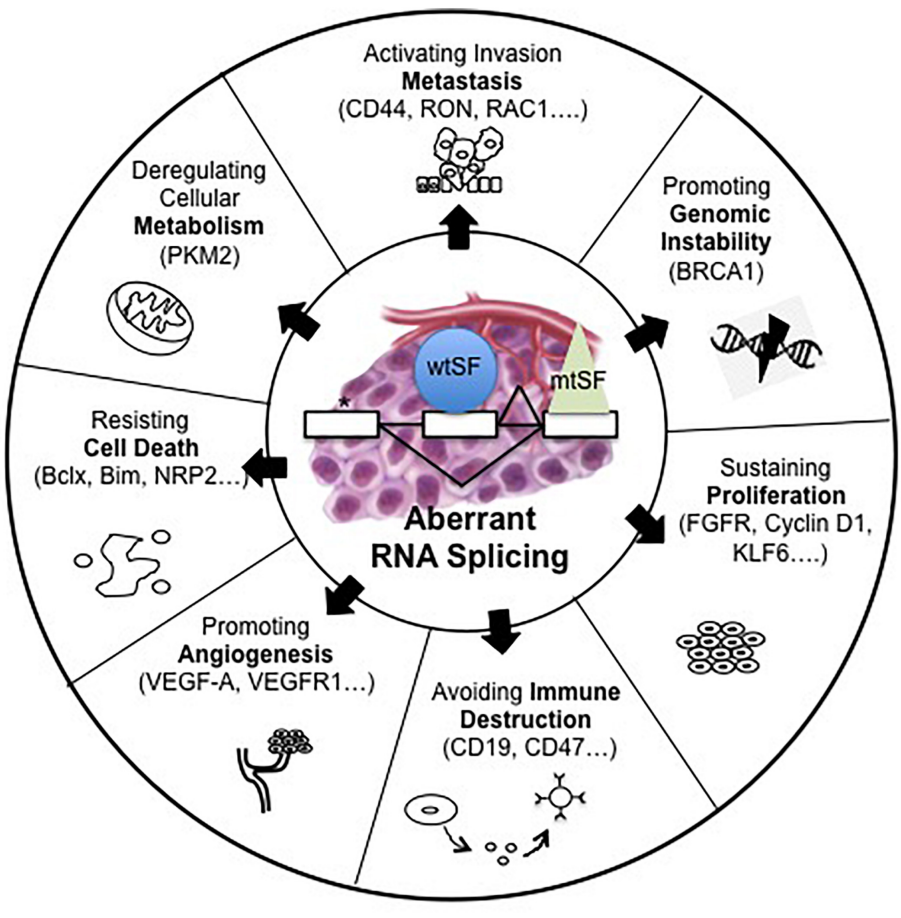

FIGURE 1 | Alterations of pre-mRNA alternative splicing in cancer. Human tumors display mutations in *cis*, which alter splice sites (*) or abnormalities in *trans* such as somatic mutations (mtSF) or deregulated expression of splicing regulatory factors (wtSF). These alterations lead to aberrant RNA splicing of numerous downstream target genes. These abnormal splicing patterns contribute to all cancer hallmarks including genomic instability, sustained proliferation, immune escape, angiogenesis, resistance to cell death, deregulated metabolism, or metastatic dissemination.

TABLE 1 | Examples of genes with splice variants associated with lung cancer progression and/or response to therapies.

Examples of genes with cancer-related splice variants	Splicing events in tumors	Functional consequences
INVOLVED IN PROGRESSION/METASTASIS		
BCL-X	Selection of a distal alternative 5' splice site	Generation of Bcl-xL, an anti-apoptotic protein overexpressed in NSCLC, which deregulates the balance between pro-apoptotic and anti-apoptotic signals
CD44	Inclusion of various exons (v1–v10)	Generation of various CD44v splice variants including CD44v8-10 with pro-tumorigenic and metastatic functions
CYCLIN D1	Retention of intron 4 due to a G/A870 polymorphism at the exon 4/intron 4 junction	Generation of cyclin D1b, a pro-tumorigenic splice variant
FGFR	Mutually exclusive exon 8 or 9	Generation of distinct extracellular Ig-like domain III with distinct affinity for FGF ligands. Induction of EMT, invasion, and motility
KLF6	Selection of a distal alternative 5' splice site	Generation of a dominant-negative splice variant termed KLF6-SV1 with a critical role in promoting cell proliferation, survival, and migration
NUMB	Inclusion of exon 9 in tumors	Reduced levels of NUMB protein expression and activation of the pro-tumorigenic NOTCH signaling
MET	Skipping of exon 14	Activation of MET kinase activity Oncogenic transformation
TP53	Selection of an alternative 3' splice site in intron 6	Generation of a p53 splice variant inducing EMT (epithelial to mesenchymal transition) markers and increasing the motility and invasive properties of lung cancer cells
NRP2	Differential alternative last exon (ALE)	In NSCLC, NRP2b contributes to the oncogenic response to TGFβ and correlates with tumor progression in patients
VEGF-A	Differential exons inclusion/skipping	Generation of various $VEGF_{xxx}$ splice variants with pro-angiogenic activity
VEGFR1	Various intron retention followed by premature polyadenylation	Production of soluble decoy sVEGFR1 acting as negative regulators of VEGFRs signaling on endothelial cells. sVEGFR1-i13 (intron 13 retention) promotes tumor cells proliferation in squamous lung carcinoma
INVOLVED IN DRUG RESPONSE/RESISTANCE		
BIM	Skipping of exon 4 due to an intronic deletion polymorphism	Generation of a BIM-γ splice variant lacking BH3 domain BIM-γexpression correlates with resistance to EGFR-TKI in NSCLC cells with EGFR mutation
KLF6	Selection of a distal alternative 5' splice site	The splice variant KLF6-SV1 promotes resistance of lung adenocarcinoma cells to platinum salts
MET	Skipping of exon 14	Confers sensitivity to MET inhibitors
NRP2	Differential ALE	NRP2b abundance is associated with acquired EGFR inhibitor resistance
VEGF-A	Selection of a distal alternative 3' splice site in last exon 8	Generation of $VEGF_{xxx}$b splice variants with anti-angiogenic function $VEGF_{xxx}$b are involved in the escape of lung cancer cells from anti-angiogenic therapies
VEGFR1	Intron 13 retention followed by premature polyadenylation	Generation of a sVEGFR1 splice variant involved in the resistance to anti-angiogenic therapies in squamous lung carcinoma

types, including lung cancer. Importantly, some of these studies unraveled splicing events with a critical role for tumor cell proliferation and/or survival in response to these therapies. Several strategies have been proposed to correct splicing. They include the use of splice-switching oligonucleotides (SSOs), which prevent the binding of splicing factors to their cognate sequence (Bauman et al., 2010). As an example, enhanced exon 6 inclusion leads to the expression of full-length MDM4 in a large number of cancers. The use of antisense oligonucleotide-mediated skipping of exon 6 decreased MDM4 abundance, inhibited tumor growth, and enhanced sensitivity to MAPK-targeting therapies (Dewaele et al., 2016). In addition, redirection of Bcl-x splicing from Bcl-xL (anti-apoptotic isoform) to Bcl-xS (pro-apoptotic isoform) induced apoptosis and increased chemosensitivity in human lung adenocarcinoma cell lines (Taylor et al., 1999). Pharmacological inhibitors targeting different components of the spliceosome machinery, either splicing factors or splicing regulators, have also been developed (Lee and Abdel-Wahab, 2016; Lee et al., 2016). Spliceostatin A, meayamycin B and sudemycins (spliceostatin A analogs), and E7107 (pladienolide analog) bind SF3B1 to prevent the formation of a U2 snRNP-SF3B1 complex with pre-mRNAs (Salton and Misteli, 2016). Regardless of splicing factor mutated status, chronic lymphocytic leukemia (CLL) and triple-negative breast cancer cells were highly sensitive to E7107, thereby identifying aberrant splicing as a targetable vulnerability in these tumor cells (Chan et al., 2017; Ten Hacken et al., 2018). Moreover, it was demonstrated that MYC drives hypersensitivity to spliceosome inhibition in various cancers, including breast cancers, suggesting a synthetic lethality relationship between oncogene activation and core spliceosome inhibition (Suda et al., 2017; Hepburn et al., 2018). These data indicated that splicing alterations could constitute one of the Achilles' heels of cancer cells, thereby supporting the idea that cancer cells would be more sensitive to spliceosome inhibition than normal cells. More recently, the role of Bcl-xL and Mcl-1 as key

determinants of cancer cell response to E7107 was shown in a large series of cancer cell lines, including NSCLC cells (Aird et al., 2019). Notably, it was found that the switch of Mcl-1 pre-mRNA splicing toward the pro-apoptotic short isoform Mcl-1_S is required for NSCLC cell death in response to E7107 treatment. These data revealed that modulation of the expression and/or of the ratio between pro- versus anti-apoptotic splice isoforms of several members of the Bcl-2 family controls cancer cell response to spliceosome inhibition. In addition, an orally available modulator of the SF3B complex, H3B-8800, was recently developed and was found to kill preferentially epithelial and hematologic tumor cells exhibiting mutations of spliceosome components (SF3B1, U2AF1, and SRSF2) (Seiler et al., 2018). H3B-8800 was granted orphan drug status by the FDA in August 2017 and is currently in clinical trials for the treatment of acute myelogenous leukemia and chronic myelomocytic leukemia. Other small-molecule inhibitors have been developed to target splicing factor kinases, such as the CDC2-like kinases (CLKs) and the serine-arginine protein kinases (SRPKs) (Lee and Abdel-Wahab, 2016; Lee et al., 2016). SRPIN340 blocks SRPK1-mediated phosphorylation of SRSF1, leading to splice switching of the pro-angiogenic $VEGF_{165}$ to the anti-angiogenic $VEGF_{165}$b splice variant of VEGF-A (Fukuhara et al., 2006; Siqueira et al., 2015). SPHINX31, a specific inhibitor of SRPK1, promoted the same splicing switch in favor of $VEGF_{165}$b and inhibited tumor growth *in vivo* (Mavrou et al., 2015). Therefore, these compounds act as anti-angiogenic molecules through modulation of VEGF-A splicing, which appears to be crucial for tumor progression. Interestingly, SRPK1 was also recently identified as a cell-essential gene in acute myeloid leukemia (AML) cell lines driven by oncogenes derived from MLL fusion genes such as MLL-AF9 and MLL-AF6 (Tzelepis et al., 2016). The same group further demonstrated that SPHINX31 induces cell cycle arrest and leukemic cell differentiation and prolongs survival of mice transplanted with MLL-rearranged AML through the modulation of the splicing of many genes with established roles in leukemogenesis such as MYB, BRD4, and MED24 (Tzelepis et al., 2018). In particular, the authors demonstrated that the switch toward the long isoform of BRD4 induced by SRPK1 inhibition affects BRD4 recruitment to the chromatin in AML cells and is detected in breast cancer cells treated with SPHINX31 (Tzelepis et al., 2018). As SRPK1 and BRD4 proteins are involved in metastasis (Alsarraj et al., 2011; van Roosmalen et al., 2015), SPHINX31 may be used to prevent tumor dissemination. Pharmacological inhibition of the CLKs inhibited the phosphorylation of SRSF1, SRSF4, and SRSF6, reduced cell proliferation, and induced apoptosis notably through the regulation of S6K alternative splicing (Araki et al., 2015). Altogether, these studies highlighted the potential of targeting splicing alterations to slow down tumor progression, either through a global approach (i.e., spliceosome inhibition) or through the use of oligonucleotide-based therapies. They also identified Bcl-2 family members or Myc oncogene as critical determinants of tumor cell susceptibility to spliceosome inhibition.

Alternative Splicing and Response to Therapies

As discussed, one of the major challenges in NSCLC patients today is to characterize the molecular mechanisms involved in the resistance to treatment in order to improve the duration of response and survival. Alterations in pre-mRNA alternative splicing have been reported during acquisition of drug resistance (Siegfried and Karni, 2018; Syafrizayanti et al., 2018; Wang and Lee, 2018). Below are some examples of splice variants that have been associated with the development of resistance in cancer (**Table 1**).

AR-v7, a constitutively activated splice variant of the androgen receptor (AR), was involved in disease progression and poor outcome upon AR-targeted therapies in castration-resistant prostate cancer patients (Antonarakis et al., 2017; Seitz et al., 2017). Accumulation of the FGFR2-IIIc splice variant of fibroblast growth factor receptor 2 (FGFR2) was associated with the progression of prostate cancer following androgen-based therapy (Sahadevan et al., 2007). In estrogen receptor-positive breast cancers, cyclin D1b, a splice variant of cyclin D1, correlated with the resistance to anti-estrogen therapy (Wang Y. et al., 2008). The BRCA1-delta11q splice variant, which lacks the majority of exon 11 of BRCA1 and consequently bypasses the inactivating germline mutations in this specific exon, promoted resistance to PARP inhibitors and cisplatin in breast cancers (Wang Y. et al., 2016). Pharmacological inhibition of the spliceosome reduced BRCA1-delta11q levels and sensitized cells harboring BRCA1 exon 11 mutations to PARP inhibitors. Aberrant splicing of BRCA2 was associated with resistance to mitomycin C (Meyer et al., 2017). In melanoma, acquired resistance to vemurafenib in patients with the BRAF(V600E) mutation was associated with the appearance of BRAF(V600E) splice variants such as those lacking exon cassette 4–8 (Poulikakos et al., 2011). Their specific decrease using splicing modulators limited the growth of vemurafenib-resistant cells or tumors (Salton et al., 2015). Other splice variants associated with acquisition of drug resistance include BCR-ABL35INS in chronic myelogenous leukemia (CML) treated with imatinib (Berman et al., 2016), a CD19 splice variant lacking exon 2 in B-cell acute lymphoblastic leukemia (B-ALLs) upon immunotherapy (Sotillo et al., 2015), and ER-alpha36, a splice variant of the estrogen receptor alpha, in ER-positive breast cancer patients upon tamoxifen therapy (Shi et al., 2009).

In lung cancer, a common deletion polymorphism in the BIM gene induces the preferential splicing of exon 3 over exon 4, leading to the synthesis of a BIM splice isoform lacking the BH3 domain (Ng et al., 2012). This polymorphism was associated with a shorter progression free survival in NSCLC patients treated with gefitinib, a tyrosine kinase inhibitor targeting epidermal growth factor receptor (EGFR-TKI) (Ng et al., 2012). Interestingly, vorinostat, an FDA-approved histone deacetylase inhibitor, corrected aberrant BIM splicing and counteracted EGFR-TKI resistance in NSCLC cell lines harboring the BIM deletion polymorphism (Nakagawa et al., 2013). Recently, we demonstrated that anti-angiogenic therapies targeting VEGFR1/VEGFR2 tyrosine kinase activity such as

sunitinib, as well as bevacizumab (Avastin^R), a monoclonal antibody against VEGF-A, increased the expression of both $VEGF_{165}b$ and sVEGFR1-i13 splice variants, which induced lung tumor escape to these therapies (Abou Faycal et al., 2018; Boudria et al., 2018). The alternative splicing of PTPMT1, a PTEN-like mitochondrial phosphatase, was also reported as a regulator of the response of lung cancer cells to radiation (sensitivity versus resistance) through the AMPK/mTOR signaling pathway (Sheng et al., 2018).

Immune checkpoint inhibitors (ICI) targeting the programmed cell death (PD-1)/programmed death ligand (PD-L1) pathways are used to counteract tumor immune escape in NSCLC patients (Meng et al., 2017). Although these treatments have radically changed the paradigm of care for these patients, a significant amount of them (9–29%) still progress, even hyper-progress, under treatment (Lo Russo et al., 2018). Up to now, the molecular mechanisms sustaining this worse response remain largely unknown. PD-1 displays alternative splice variants through differential exon skipping (Nielsen et al., 2005; Zhu and Lang, 2017). The PD-1 Deltaex3 splice variant does not have exon 3 and encodes a soluble isoform (sPD-1) (instead of a membrane-bound protein), which was not detectable in healthy individuals (Nielsen et al., 2005). Interestingly, increased sPD-1 levels were reported in NSCLC patients treated with erlotinib and were associated with prolonged progression-free and overall survival (Sorensen et al., 2016). Another PD-L1 splice variant that results from exon 2 skipping and encodes a soluble PD-L1 (sPD-L1), instead of membrane-bound PD-L1 (mPD-L1) isoform, was also described (He et al., 2005). In NSCLC patients, the expression of this circulating plasmatic sPD-L1 was up-regulated compared to the healthy controls and was associated with a bad prognosis (Cheng et al., 2015; Zhang et al., 2015; Okuma et al., 2017). As sPD-L1 may also be generated in response to mPD-L1 cleavage by metalloproteases, the contribution of alternative splicing in the biogenesis of sPD-L1 in lung cancer patients remains to be determined. Whether variations in the level of PD-1 or PD-L1 splice variants could account for distinct ICI response in lung cancer patients also remains to be tested.

As a whole, these data clearly define a link between dysregulated alternative splicing processing events and resistance to various therapies in cancer, including lung cancer. In this setting, it remains to be determined whether resistance could be reverted by using spliceosome inhibitors, either alone or in combination. In agreement with such a possibility, E7107 sensitized leukemic cells to venetoclax, a Bcl-2 inhibitor, by switching the splicing of Mcl-1 from Mcl-1_L (anti-apoptotic) to Mcl-1_S (pro-apoptotic) splice variant (Ten Hacken et al., 2018). The combination of SPHINX31 with an epigenetic drug, namely, i-BET-151, a BET inhibitor, showed synergistic effects against AML without noticeable toxicity in mice, suggesting the possible clinical use of SRPK1 inhibitors in combination with bromodomain inhibitors (Tzelepis et al., 2018). In addition, pladienolide B demonstrated a greater efficacy in lung cancer cell lines established from patients with prior chemotherapy (Suda et al., 2017) and significantly increased the sensitivity of various cancer cells to cisplatin (Anufrieva et al., 2018). Altogether, these studies highly support the idea that spliceosome inhibitors could be promising molecular target drugs in combination with conventional/targeted therapies.

RNA Splice Variants: Predictive Biomarkers in Liquid Biopsies of Lung Cancer Patients?

As circRNAs, splice variants may be promising biomarkers for prognosis and post-treatment follow-up of cancer. Up to now, their detection has been done mainly in tumor tissue biopsies using either RNA sequencing and various PCR techniques for validation using primers allowing to distinguish between splice variants, or immunohistochemistry using specific antibodies of each splice variant. CTCs have also been used as a source to detect RNA splice variants. As an example, up-regulation of the splice variant delta5 of the estrogen receptor ESR1 was reported in CTCs isolated from whole blood of metastatic breast cancer patients compared to healthy blood donors (HBDs) (Beije et al., 2018). However, these analyses were complex because a subset of HBDs and healthy breast tissue also expressed this splice variant (Poola and Speirs, 2001). In contrast, full-length transcript and splice variants of the AR are typically absent in whole blood of HBDs (Onstenk et al., 2015). In metastatic prostate cancer, the expression of the AR splice variant V7 in CTCs was strongly associated with resistance to endocrine agents (Antonarakis et al., 2014, 2015). These results led to the development of a specific test to detect AR-V7 in CTCs from blood samples.

In contrast, the detection of circulating RNA splice variants in liquid biopsies (plasma, serum) is much more complex. Circulating free RNA degrades very quickly, and plasma samples need to be processed rapidly after blood extraction. The usual procedure is to freeze the sample at -80°C in an RNA preservative solution such as Trizol. In addition, the expression of splice variants by normal blood cells such as leukocytes may complicating the analyses. Nevertheless, some studies are promising. Seitz et al. (2017) showed that AR-v7 mRNA levels in whole blood of patients with castration-resistant prostate cancer could predict a poor outcome in response to androgen receptor-targeted therapies (abiraterone, enzalutamide). Somatic mutations affecting splice sites of exon 14 of the MET gene (METex14) have been reported in NSCLC (Ma et al., 2003; Kong-Beltran et al., 2006). These METex14 alterations induced constitutive activation of MET kinase activity (Frampton et al., 2015), and MET-targeted therapies are now used in MET14-positive NSCLC patients (Onozato et al., 2009; Paik et al., 2015). Recently, METex14 splice variant was detected in platelet-derived RNA from a NSCLC patient who partially responded to crizotinib (Aguado et al., 2016). Although this result has to be confirmed, this suggests that detection of METex14 splice variants in liquid biopsies could help to select good responders to MET-targeted therapies.

As a whole, it is clear that the detection of RNA splice variants in liquid biopsies, despite remaining a challenge, is a promising field.

CONCLUSION AND PERSPECTIVES

There is a crucial need of both sensitive and specific cancer biomarkers, including proteins, DNAs, or RNAs, for early diagnosis, prognosis, and patient follow-up. Tissue biopsies have been widely used as a reservoir of these biomarkers. However, tissue biopsies are invasive procedures and some patients are not eligible, especially for repeated collection. In addition, tissue biopsies do not mirror tumor heterogeneity and metastatic burden of most advanced tumors. In contrast, liquid biopsies are less invasive than tissue biopsies, and circulating biomarkers more likely reflect intratumoral heterogeneity and dissemination. However, we are facing other challenges mostly involving the way to collect body fluids, the choice of biological materials to be analyzed (saliva, urine, whole blood, circulating cells, and platelet-enriched or platelet-deprived plasma), the selection of appropriate controls, and the standardization and optimization of pre-analytical and analytical procedures. It is clear that improvement of all these steps will be required in the future if we want to use liquid biopsies as a surrogate of tissue biopsies to detect and quantify accurate cancer biomarkers.

We have to select the most accurate genome-wide transcriptomic analyses to profile circRNAs and RNA splice variants in tumor patients. In this setting, we have to consider the reproducibility, the sensitivity, and the cost of these analyses if we want to translate them into the clinic. Deep RNA-sequencing allows the identification of previously known as well as unknown circRNAs and RNA splice variants, and sensitivity can be improved by increasing sequencing depth, which may also increase the cost. RNA-seq allows a better determination of gene/transcript concentration. However, for circRNAs, the detection efficiency is limited, as only 0.1% reads from RNA-seq cover the head-to-tail junction (Szabo and Salzman, 2016). Li S. et al. (2018b) recently compared the circRNA detection efficiency of RNA-Seq (20 million sequencing reads) and a circRNA microarray containing probes targeting back-splice site of 87,935 circRNA sequences covering most of the circRNAs represented in circBase. The results showed that the microarray is more efficient than RNA-Seq for circRNA profiling. CircRNA microarray detected about 80,000 circRNAs in matched cervical tumors and normal tissues from 10 patients with about 25,000 circRNAs differentially expressed in tumors. Around 18,000 circRNAs were also detected in cell-free plasma samples of 21 patients with cervical cancer, and approximately 2,700 of them were differentially expressed after surgical tumor removal (Li S. et al., 2018b). Therefore, besides RNA-Seq, these data suggested that circRNA microarrays could be an efficient and sensitive tool for the profiling of specific circRNAs in tumor liquid biopsies. Moreover, Romero et al. (2018) compared the performance of RNA-Seq and splice junction arrays for the analysis of transcript splicing events in breast cancer cell lines treated with a drug that affects splicing. They observed a high degree of coherence between the two technologies, with the detection power of the junction array being equivalent to RNA-Seq with up to 60 million reads. Nazarov et al. (2017) compared RNA sequencing (Illumina 2000 RNA-seq) and transcriptome arrays (Affymetrix HTA 2.0) in patient samples derived from normal lung epithelium and squamous cell lung carcinoma. The analysis of alternative splicing, more specifically differential exon usage, produced discordant results between the two platforms, with higher stochastic variability and insufficient reads for many exons and exon junctions in RNA-Seq. Therefore, microarray techniques could also constitute a faster and cheaper alternative to RNA-sequencing for RNA splice variant profiling, especially to compare relatively large groups of samples or to focus on well-annotated transcriptional regions and already known RNA splice variants.

The interest of large-scale RNA analyses in body fluids is to establish the landscape of cell-free RNA transcriptome, to provide new insights into the temporal dynamics of circulating RNAs, and to estimate the relative contribution of tissues to the pool of circulating RNAs through the analyses of tissue-specific genes. In the RNA field, RNA splice variants and circRNAs have recently emerged as cancer biomarker candidates, mainly because thousands of them are aberrantly expressed in various cancer types compared to normal tissues. As a consequence, many papers have proposed specific RNA splice variants or circRNAs as cancer biomarkers or as useful tools for prediction of patients' outcome. However, in most of the cases, the biological consequences of their deregulation remain unknown. In addition, the vast majority of circRNA biomarker studies published to date are retrospective. It is therefore hard to decipher whether these circRNAs and RNA splice variants belong to a common driving mechanism of oncogenesis or are just common by-product/end-product of oncogenesis. Very recently, using an ultra-deep non-poly-A RNA-seq on 144 localized prostate tumors, Chen et al. (2019) showed that the abundance of both global and specific circRNAs is associated with clinical outcome. These results suggested that some circRNAs play oncogenic roles. As a whole, these data highlight the need to characterize the functions of these aberrantly expressed RNA splice variants or circRNAs in order to understand their contribution to tumor progression and/or tumor escape to therapies. Moreover, as RNA splice variants or circRNAs could be highly expressed in some blood cells such as platelets, their detection in whole blood could only reflect the level of circulating cells. Therefore, a careful comparison of their expression level between healthy donors and cancerous patients, a thorough description of serum/plasma transportation mechanisms, and the study of the origin of these RNA splice variants or circRNAs with accurate methods should be considered as preliminary steps before proposing them as relevant cancer biomarkers. Keeping these precautions in mind, it is likely that some of these circulating RNAs will emerge as relevant cancer biomarkers in the near future and enter into clinical practice.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Role of lncRNAs in the Distant Metastasis of Breast Cancer

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Breast cancer (BC) remains the most frequently diagnosed cancer worldwide. Among breast cancer patients, distant metastasis and invasion is the leading cause of BC related death. Recently, long non-coding RNAs (lncRNAs), which used to be considered a genetic byproduct (owing to their unknown biological function), have been reported to be highly implicated in the development and progression of BC. In this review, we produce a summary of the functions and mechanisms of lncRNAs implicated in the different distant metastases of BC. The functions of lncRNAs have been divided into two types: oncogenic type and tumor suppressor. Furthermore, the majority of them exert their roles through the regulation of invasion, migration, epithelial—mesenchymal transition (EMT), and the metastasis process. In the final part, we briefly addressed future research prospects of lncRNAs, especially the testing methods through which to detect lncRNAs in the clinical work, and introduced several different tools with which to detect lncRNAs more conveniently. Although lncRNA research is still in the initial stages, it is a promising prognosticator and a novel therapeutic target for BC metastasis, which requires more research in the future.

Keywords: long non-coding RNA, breast cancer, metastasis, invasion, mechanism

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INTRODUCTION

In past years, breast cancer (BC) has always been the most frequently diagnosed cancer worldwide, and is also the major reason for cancer-related mortality in women. It is estimated that there will be 266,120 new cases of female breast cancer and an estimated 40,920 people will die of this disease in the United States in 2018 (https://www.cancer.gov/types/common-cancers). Among these patients, distant metastatic invasion has been proposed as the leading cause of death (1–4). According to an estimation, 20–30% of patients with breast cancer will develop metastasis following diagnosis (5).

Long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides in length, are one subtype of RNA transcripts which used to be considered a genetic byproduct (owing to their unknown biological function) (6). In recent years, the human genome has been gradually transcribed based on the development of DNA sequencing technologies, and it is unbelievable that more than 98% of genes of the whole human genome are non-encoding, receiving a considerable amount of concern in recent years. Thanks to biological techniques and high-throughput

sequencing, such as SHAPE probing (7), ChIRP (8), and CHART (9), which can be used to reveal RNA structures and RNA–RNA, RNA–DNA, and RNA–protein interactions, researchers have uncovered a great amount of lncRNAs in past years, which have played significant roles in various biological processes, especially in the progression of malignant tumors (10, 11). LncRNAs can be classified into five categories—(1) sense, (2) antisense, (3) bidirectional, (4) intronic, or (5) intergenic—with respect to the nearest protein-coding transcripts (12). A diversity of malignant tumors has been reported to be involved with lncRNAs. In these studies, lncRNAs seem to play an indispensable role in the progression of cancer, both in the development of the primary tumor and in the metastatic procedure (13, 14).

Numerous lncRNAs have been reported to be highly implicated in the development of BC (15–18), which could be categorized into two types: oncogenic type and tumor suppressor. Regardless of whether they act as a promoter or inhibitor during the progression of BC, the mechanism generally covers the following aspects: influence proliferation, invasion, apoptosis and drug resistance of BC cells. However, the molecular mechanisms in respect of how lncRNAs come into play in the distant metastasis of breast cancer remain insufficient. The functions and mechanisms of these lncRNAs—such as MALAT1 (19), HOTAIR (20) and NEAT1 (21)—have already been widely accepted regarding their interaction in the metastasis of BC. On account of the significant role that lncRNAs have played in BC metastasis, it is an urgent requirement to explore them as predictors for prognosis and as novel targets for therapy in BC patients.

With the inactivation of embryo dedifferentiation, epithelial–mesenchymal transition (EMT) increases the movement and proliferation of cancer cells (22). Transient EMT is thought to be the first necessary step in metastatic processes (23), which contributes to the migration and invasion of cancer cells, so that cancer cells leave the primary focus and form distant metastasis (24). In recent years, some researchers have indicated that EMT plays an indispensable role in the potential mechanism leading to the distant metastasis of BC (25).

In this review, we produce a summary of the functions and mechanisms of lncRNAs implicated in the different distant metastases of BC (**Table 1**), especially via the EMT program (**Table 2** and **Figure 1**).

LncRNAs IN THE DISTANT METASTASIS OF BREAST CANCER

LncRNAs Implicated in the Lung Metastasis of BC

MALAT1

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was first found in non-small cell lung cancer (NSCLC) patients. Functioning as a poor prognosticator, its overexpression could be predictive of a higher stake related to distant metastasis in a series of cancers such as lung cancer, osteosarcoma, colorectal cancer, and especially in non-small cell lung cancer patients (54, 55). Zhang et al. have concluded that MALAT1 is actively involved in multiple physiological processes, such as myogenesis, alternative splicing, synapse formation, and epigenetic modification of gene expression, as well as in multiple pathological conditions (56).

In the past few decades, many *in vitro* and xenograft researches have reported the underlying functions of MALAT1 in regulating invasion, migration, EMT and the metastasis procedure of a diversity of malignancies, and have indicated inconsistent functions of MALAT1 with respect to the growth and progression of tumor cells (57–59). Experiments both *in vitro* and *in vivo* have revealed that MALAT1 is a proliferation promoter, as well as accelerating tumor development and metastasis in triple-negative breast cancer (TNBC). Moreover, it is even negatively correlated with the prognosis of breast cancer patients with an HER-2 positive ER-negative subtype or triple-negative subtype (60). Another study has demonstrated that 17b-Estradiol (E2) with a high level of concentration may inhibit cell growth, invasion and metastasis; in the meantime, the level of MALAT1 is reduced as well either in MCF7 cell lines (Luminal A) or in MDA-MB-231 cell lines (TNBC). Similar effects could be achieved through downregulated MALAT1, so E2 may influence tumor cells through regulating the lncRNA MALAT1 (61).

Xu et al. found that MALAT1 was downregulated in breast tumor cell lines and cancer tissue, and downregulation of MALAT1 in breast cancer cell lines through the activation of phosphatidylinositide-3 kinase-AKT (PI3K-AKT) pathways later results in EMT (58). Recently, MALAT1 was also considered a proinflammatory factor which regulated the lipopolysaccharide (LPS)-induced inflammatory response (62) and EMT process of breast cancer cells (63, 64). The upregulation of EMT-related protein (MMP-9 and vimentin) is associated with NF-κB, which would be inhibited after decreasing the expression of MALAT1 (27). CD133 (PROMININ1), which is one of the general cancer stem cell (CSC) markers, has been reported to possess the ability to facilitate EMT in breast cancer and other malignant tumors (65), and Latorre et al. recently showed that the failure to form or stabilize a repressive complex consisted of MALAT1 and the RNA-binding protein HuR in breast cancer upregulates CD133 and leads to an EMT-like program with the increase of N-cadherin (44).

Some studies showed that MALAT1 inhibited the expression of E-cadherin and induced the expression of vimentin at mRNA and protein levels, while miR-1 inhibited the expression of vimentin and MMP-9 while enhancing the expression of E-cadherin in Western blot results, which can be summarized as MALAT1 and miR-1 having opposite effects on the migration and invasion of breast cancer cells. In conclusion, MALAT1 acts as ceRNA of Cdc42 by binding to miR-1 and then leads to EMT in human breast cancer cell lines (47). miR-204 expression was downregulated by MALAT1 via acting as an endogenous sponge. MiR-204 inhibited the expression of ZEB2 by binding to the non-coding region of ZEB2 3-UTR. Therefore, MALAT1 regulated the miR-204/ZEB2 axis in breast cancer. In view of the fact that ZEB2 is a key factor in EMT, it was speculated that MALAT1 may promote cell metastasis and result in an EMT phenotype via the miR-204/ZEB2 axis (46).

TABLE 1 | LncRNAs implicated in invasion, migration, EMT and metastasis of BC.

LncRNA	Description	Classification	Metastasis	Injection place	Function	Functions in BC	Potential mechanism	Ref.
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	Intergenic	Lung	Tail vein	Tumor suppressor	↓Invasion, migration, metastasis	MALAT1 suppresses metastasis in a TEAD-dependent manner, which associates and inhibits the prometastatic transcription factor TEAD through binding to its target gene promoters and co-activator YAP.	(26)
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	Intergenic	Lung	Coupled abdominal mammary glands	Oncogenic	↑Invasion, migration, metastasis, EMT	Knocking down of MALAT1 in the 4T1 cells, lung metastasis and inflammatory responses were significantly reversed. TNF-α level in the supernatants was decreased sharply, accompanied by the weakened ability of invasion and migration induced by LPS.	(27)
HOTAIR	Homeobox transcript antisense RNA	Antisense	Lung	Tail vein	Oncogenic	↑Invasion, metastasis	HOTAIR expression in epithelial carcinoma cells led to genome-wide re-targeting of PRC2 to an occupancy pattern more like embryonic fibroblasts, resulting in gene expression, increased cancer metastasis as well as invasiveness depend on PRC2.	(20)
HOTAIR	Homeobox transcript antisense RNA	Antisense	Lung	Mammary fat pads	Oncogenic	↑Invasion, migration, metastasis, EMT	CAFs promoted the metastatic activity of breast cancer cells by activating the transcription of HOTAIR via TGF-β1 secretion	(28)
NEAT1	Nuclear enrich abundant transcript 1	Intergenic	Lung	Left abdominal mammary fat pad	Oncogenic	↑Invasion, dissemination, metastasis, EMT	NEAT1 acts as a pivotal part in BC metastasis via the ERa-NEAT1-FOXN3/NEAT1/SIN3A-GATA3 axis.	(29)
NEAT1	Nuclear enrich abundant transcript 1	Intergenic	Lung	Tail vein	Oncogenic	↑Invasion, EMT, metastasis,	LncRNA NEAT1 induced EMT through the miR-211/HMGA2 axis.	(21)
Linc-ROR	Long Non-Coding RNA Reprogramming	Intergenic	Lung	Lateral tail veins	Oncogenic	↑Invasion, migration, metastasis, EMT	Linc-ROR functions as an important regulator of EMT and can promote breast cancer progression and metastasis through regulation of mi-205.	(30)
UCA1	Urothelial carcinoma-associated 1	Intergenic	Lung	Mammary fat pads	Oncogenic	↑Invasion, migration, metastasis, EMT	AC026904.1 and UCA1 could cooperatively upregulate Slug expression at both transcriptional and post-transcriptional levels, exerting key roles in TGF-β-induced EMT.	(31)
TINCR	Terminal differentiation-induced non-coding RNA	Intronic	Lung	Lateral tail vein	Oncogenic	↑Invasion, migration, metastasis, EMT	TINCR located in the cytoplasm of BC cells and have the ability to sponge miR-125b, upregulating the expression of miR-125b-targeted Snail-1 could reverse inhibited invasion, EMT, and migration resulted from silencing of TINCR.	(32)
BORG	BMP/OP-Responsive Gene	-	Lung	Lateral tail vein	Oncogenic	↑Invasion, migration, metastasis, EMT	BORG induces the metastatic colonies of potent BC cells by activating the transcriptional repressive activity and localization of TRIM28, which combines with BORG and leads to a great amount of changes in cancer progression.	(33)
LincIN	A long intergenic non-coding RNA between ITGB1 and NRP1	Intergenic	Lung	Tail vein	Oncogenic	↑Invasion, migration, metastasis,	LincIN exerts a critical role in translational alterations by regulating p21 as well as interacting with NF90, consequently leads to invasiveness and metastasis of BC cells.	(34)
ANCR	Anti-differentiation ncRNA	Intergenic	Lung	Tail vein	Tumor suppressor	↓Invasion, migration, metastasis, EMT	Linking ANCR interaction with EZH2 to promote its phosphorylation that facilitates EZH2 degradation and suppresses breast cancer progression.	(35)

(Continued)

TABLE 1 | Continued

LncRNA	Description	Classification	Metastasis	Injection place	Function	Functions in BC	Potential mechanism	Ref.
Lnc015192	-	-	Lung	Tail vein	Oncogenic	↑Invasion, migration, metastasis, EMT	Lnc015192 and Adam12 all have the function to promote metastasis of BC and maybe partly through sponging miR-34a via the ceRNA mechanism.	(36)
LINC01638	Long intergenic non-protein coding RNA 1683	Intergenic	Lung	Tail vein xenograft	Oncogenic	↑Invasion, metastasis, EMT	LINC01638 interacts with c-Myc to prevent SPOP-mediated c-Myc ubiquitination and degradation. C-Myc transcriptionally enhances MTDH (metadherin) expression and subsequently activates Twist1 expression to induce EMT.	(37)
NKILA	NF-KappaB Interacting lncRNA	-	Lung and liver	Tail vein	Tumor suppressor	↑Apoptosis, ↓invasion	NKILA Inhibits NF-kB-mediated breast cancer metastasis	(38)
NKILA	NF-KappaB interacting lncRNA	-	Lung and liver	Mammary fat pads	Tumor suppressor	↓Invasion, migration, metastasis, EMT	TGF-β activates the NF-κB pathway. Inhibition of NF-κB signaling markedly abrogates TGF-β-induced EMT, the NKILA-mediated negative feedback affects TGF-β-induced NF-κB activation.	(39)
ARNILA	AR negatively induced lncRNA	-	Lung and liver	Tail vein	Oncogenic	↑Invasion, migration, metastasis, EMT	ARNILA functioned as a ceRNA for miR-204 to facilitate expression of its target gene Sox4, thereby promoting EMT, invasion and metastasis of TNBC.	(40)
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	Intergenic	Lung and liver	An orthotopic injection	Oncogenic	↑Invasion, migration, metastasis	MiR-1 inhibits metastasis of BC cells by targeting MALAT1	(19)
Lnc-BM	LncRNA associated with BCBM.RP11-355I22.7 (AK055647)	-	Brain	Intracardiac injection or intra-arterial injections	Oncogenic	↑Invasion, migration, vascular co-option	Lnc-BM drove STAT3-dependent expression of CCL2and ICAM1, which acts as a mediator in the process of recruitment of macrophages and vascular co-option in the cerebrum. Macrophage which have been recruited inversely produced IL-6 and oncostatin M, thereby further activating the Lnc-BM/JAK2/STAT3 pathway and promoting BM.	(41)
XIST	X-inactive–specific transcript	Intergenic	Brain and lung	Left cardiac ventricle	Tumor suppressor	↓Invasion, migration, metastasis, EMT	Decreased expression of XIST stimulated EMT and activated c-Met via MSN-mediated protein stabilization, which resulted in the promotion of stemness in the tumor cells.	(42)
HOTAIR	Homeobox transcript antisense RNA	Antisense	Lungs, kidneys and adrenalin glands	Tail vein	Tumor suppressor	↓Invasion, migration, metastasis, EMT	MiR-7 inhibits EMT and metastasis through downregulation of the STAT3 pathway. MiR-7 expression is suppressed by HOTAIR.	(43)

LncRNA, long non-coding RNA; EMT, epithelial-mesenchymal transition; BC, breast cancer; Ref, reference.

As part of *in vivo* studies, Zhang et al. generated mice models with a deleted-3-kb genomic site encompassing the 5′end of lncRNA MALAT1 as well as its promoter region (66). Another study reported that the systemic knockdown or genetic loss of MALAT1 in the MMTV (mouse mammary tumor virus)-PyMT (polyomavirus middle T antigen) (67) rats model led to slower growth of tumor cells as well as a reduction in lung metastases (68). They found a diminishment of branching morphogenesis in Her2/neu-amplified and MMTV-PyMT tumor tissues and cell migration accompanied by an enhancement of cell adhesion in MALAT1-loss cells; however, the potential mechanism regarding the reduced metastasis remained unclear. It is considered that this MALAT1-loss model is usually accompanied by the upregulation of substantial adjacent genes of MALAT1, including Neat1, Tigd3, Frmd8, Ehbp1l1, and so on (66). In order to explore whether this high level of expression was caused by the deletion of MALAT1 or the loss of regulatory sequences for its adjacent genes, Kim et al. adopted a MALAT1-knockout animal model wherein a transcriptional terminator was inserted 69-bp downstream of the transcriptional start region of MALAT1. When the expression of MALAT1 was restored in breast tumor tissue, the distant metastasis in the lung was reduced. Besides, they proposed that MALAT1 suppresses metastasis in a TEAD-dependent manner, which associates and inhibits the

TABLE 2 | LncRNAs implicated in the EMT program of BC.

lncRNA	Publication time	EMT markers	Potential mechanism	References
MALAT1	2016	↑N-cadherin	The failure to form or stabilize a repressive complex consisted of MALAT1 and HuR upregulates CD133 and lead to an EMT-like program	(44)
MALAT1	2015	↑N-cadherin	Downregulation of MALAT1 through the activation of PI3K-AKT pathways later results in EMT	(45)
MALAT1	2018	↑Vimentin, MMP9	The upregulation of EMT-related protein(MMP-9 and vimentin) is associated with NF-κB, which would be inhibited after decreasing the expression of MALAT1	(27)
MALAT1	2017	↓E-cadherin, ↑Vimentin, N-cadherin	MALAT1 may promote cell metastasis and result in EMT phenotype via the miR-204/ZEB2 axis	(46)
MALAT1	2016	↓E-cadherin, ↑Vimentin, MMP9	MALAT1 acts as ceRNA of Cdc42 by binding to miR-1 and then lead to EMT.	(47)
HOTAIR	2018	↓E-cadherin,↑Vimentin, β-catenin	TGF-β1 secreted by CAFs, activates TGF-β1/SMAD pathway leading to the positively-regulation of HOTAIR transcription and histone modification of CDK5 signaling pathway.	(28)
HOTAIR	2013	↑Vimentin, fibronectin	HOTAIR upregulated by TGF-β1 acts as a key regulator that controls the multiple signaling mechanisms involved in EMT.	(48)
NEAT1	2017	↓E-cadherin, ↑Vimentin, N-cadherin	lncRNA NEAT1 can induce EMT through the miR-211/HMGA2 axis.	(49)
NEAT1	2017	↓E-cadherin, ↑Vimentin, Fibronectin	FOXN3-NEAT1-SIN3A complex promotes EMT by inhibiting the transcription of downstream target genes GATA3 and TJP1.	(29)
NEAT1	2016	↓E-cadherin, ↑N-cadherin	Increased expression of NEAT1 stimulated EMT and the underlying mechanism is not clear.	(18)
linc-ROR	2017	↓E-cadherin	Downregulation of lncRNA-ROR can inhibit EMT by increasing the expression of a negative regulator miR-205-5p and reducing the expression of ZEB1 and ZEB2 which both capable of binding to E-boxes in the E-cadherin promoter	(50)
linc-ROR	2014	↑E-cadherin, ↑Vimentin, Fibronectin, N-cadherin, aSMA	Linc-ROR overexpression prevents the degradation of mir-205 target genes, including the EMT inducer ZEB2.	(30)
UCA1	2016	↓E-cadherin, ↑ N-cadherin, Vimentin, Snail	UCA1-induced EMT at least partly via activating the Wnt/β-catenin signaling pathway	(51)
UCA1	2018	↓E-cadherin, ↑N-cadherin, Fibronectin	AC026904.1 and UCA1 cooperatively upregulate Slug expression at both transcriptional and post-transcriptional levels.	(31)
TINCR	2019	↓E-cadherin, β-catenin, ↑Vimentin, N-cadherin	TINCR induced by acetylation of H3K27 up-regulates miR-125b, and promotes the EMT process by increasing the expression of Snail-1.	(32)
ANCR	2016	↓E-cadherin, ↑Vimentin	Through influencing the stability of EZH2, ANCR negatively regulated the process of EMT.	(52)
ANCR	2017	↓E-cadherin, ↑N-cadherin, Vimentin	ANCR, as a new downstream molecule of TGF-β, plays an important role in TGF-β1-induced EMT by reducing the expression of RUNX2.	(21)
lnc015192	2018	↓E-cadherin, ↑N-cadherin, Vimentin	lnc015192 could be used as the ceRNA of miR-34a to modulate Adam12, but the detailed mechanism of EMT was not defined.	(36)
LINC01638	2018	↓E-cadherin, ↑Vimentin	LINC01638 interacts with c-Myc to inhibit SPOP-mediated c-Myc degradation and ubiquitination. C-Myc promotes MTDH expression and therefore stimulates Twist1 expression to result in an EMT program.	(37)
lncRNA-HIT	2015	↓E-cadherin, ↑Vimentin	LncRNA HIT can be induced by TGF-β and play a critical role in TGF-β-induced EMT	(53)
NKILA	2018	↓E-cadherin, ↑Vimentin	TGF-β enhances the expression of NKILA, thus inhibiting the overactivation of NF- κB and TGF-β-induced EMT	(39)
ARNILA	2018	↓E-cadherin, ↑N-cadherin	ARNILA promoted EMT by competitively binding to miR-204, leading to the upregulation of Sox4.	(40)
XIST	2018	↓E-cadherin, ↑Vimentin	Decreased expression of XIST stimulated EMT and the underlying mechanism is not clear.	(42)

prometastatic transcription factor TEAD through binding to its target gene promoters and coactivator YAP (26).

The opposite observation results from disparate studies suggested the complicacy of MALAT1 in BC, which may be according to the specific tumor subtypes or different cell types.

HOTAIR

HOTAIR, which is an lncRNA in the mammalian HOXC locus that targets and binds to polycomb repressive complex 2 (PRC2) to the HOXD locus, is located on a disparate chromosome (69).

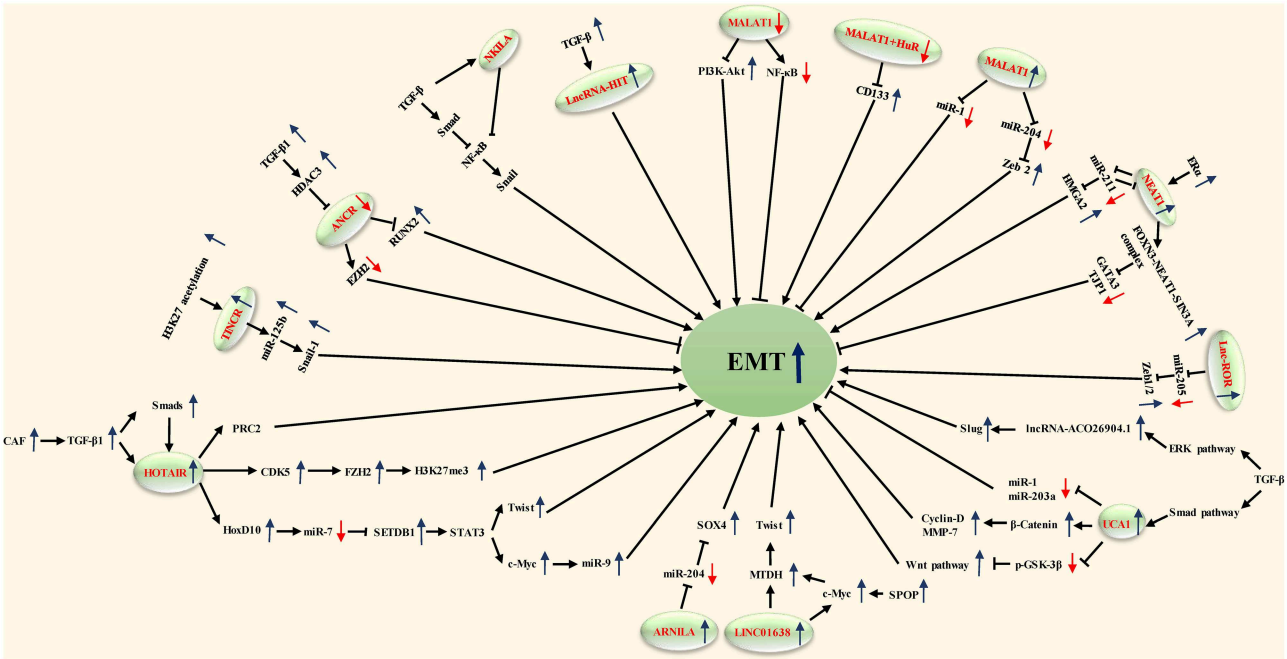

FIGURE 1 | LncRNAs that are known to regulate EMT processes and their validated targets.

HOTAIR expression is upregulated in breast cancer tissue and its metastasis, and could also predict the eventual metastasis and death according to different expression levels. LncRNA HOTAIR has been proven through interacting with PRC2, which can promote breast cancer progression (20). For example, the interaction of HOTAIR with PRC2 or LSD1 can be inhibited by HOTAIR targeting small molecular inhibitors, thereby reducing the metastasis of breast cancer (70).

Some findings suggest that HOTAIR is involved in the activation of the genetic process that promotes EMT. Padua Alves et al. have shown that HOTAIR is an important regulator of EMT-related genes in breast cancer cells, and transforming growth factor-β1 (TGF-β1) supports this role of HOTAIR (48). TGF-β-1 induced the upregulated expression of HOTAIR, which may be a central event in promoting PRC2 to inhibit E-cadherin gene expression. This hypothesis is supported by other extended analyses of gene expression profiles, PRC2 occupation, and H3K27me3 (20). In these analyses, they observed the activation of EMT in cells overexpressing HOTAIR. Other experiments generally support the view that TGF-β1 secreted by cancer-associated fibroblasts (CAFs) activates the TGF-β1/SMAD pathway in breast cancer cells, whereby leading to the positive regulation of HOTAIR transcription and histone modification of the CDK5 signaling pathway. Therefore, EMT can be induced to promote the progression and lung metastasis of breast cancer *in vivo* (28). Moreover, HOTAIR can inhibit the expression of miR-7. In turn, can significantly induce the expression of E-cadherin, and reverse EMT by downregulating the STAT3 pathway (43).

NEAT1

Nuclear-enriched abundant transcript 1 (NEAT1) is a sub-nuclear structure localized exclusively to paraspeckles (71). Its function is that of modulating RNA splicing as well as transcription. It has been proposed that NEAT1 functions as an oncogenic lncRNA in various kinds of malignancies, such as BC, and it is suggested to induce EMT in cancer progression (72).

Several studies have reported that lncRNA NEAT1 accelerates the proliferation and progression of malignancies. EMT is one of the essential contributors to invasion and metastasis in breast carcinoma (25). Zhang et al. found that the expression of EMT markers was altered according to the expression of lncRNA NEAT1. The expression of E-cadherin was increased and N-cadherin was decreased by inhibiting lncRNA NEAT1, suggesting that lncRNA NEAT1 might regulate EMT in BC; however, the underlying mechanism remains unclear (18).

HMGA2 has been reported to regulate EMT transcription factor (EMT-TF) networks such as Snail1, Snail2, ZEB1, ZEB2, and Twist1 (58). There was mutual inhibition between NEAT1 and miR-211. In addition, EMT inducer HMGA2 was identified as a downstream target of miR-211. This concluded that lncRNA NEAT1 can induce EMT through the miR-211/HMGA2 axis (21). As part of an *in vivo* experiment, cells transduced with sh-NEAT1 were inoculated into animal models through the tail vein, and it was found that compared with the control group, the experimental group generated smaller distant metastatic colonies in the lung, meaning that inhibiting the activity of lncRNA NEAT1 reduced the metastatic ability of cancer cells *in vivo* (21).

FOXN3 is a transcription inhibitor associated with SIN3A repressor complexes in estrogen receptor-positive (ER+) cells.

It has been shown that an FOXN3–NEAT1–SIN3A complex promotes the invasion of breast cancer cells and EMT *in vitro* and the proliferation and metastasis of breast cancer *in vivo* by inhibiting the transcription of downstream target genes GATA3 and TJP1. After inoculating the engineered MCF-7 breast cancer cells into the left abdominal mammary fat pad, it was found that overexpression of either NEAT1 or FOXN3 efficiently promoted lung metastasis in animal models (29).

Linc-ROR

It has been reported for lncRNA-regulator of reprogramming (lincRNA-ROR) that the expression is upregulated in several different solid tumors, which suppresses the invasion of a tumor through reducing the EMT marker's expression (73). Additionally, lincRNA-ROR also potently promotes the invasion and distant metastasis ability of BC through the EMT program.

Hou et al. first identified the role of linc-ROR in the control of EMT and metastasis in breast cancer cells. Linc-ROR is associated with miRNPs and acts as an endogenous RNA competing with mi-205. Specifically, linc-ROR overexpression prevents the degradation of mir-205 target genes in breast cancer cells, including the EMT inducer ZEB2. Therefore, linc-ROR is an important regulator of EMT and promotes the progression and metastasis of breast cancer by regulating miRNAs (30). It has been verified that in MDA-MB-231 cells, the downregulated expression of lncRNA-ROR can inhibit the EMT of breast cancer by increasing the expression of negative regulator miR-205-5p and reducing the expression of ZEB1 and ZEB2, key EMT-TFs that, more than others, play a central role in controlling EMT activation (74), both of which include two zinc finger domains capable of binding to E-boxes in the gene promoter region, such as the E-cadherin promoter (50).

UCA1

Urothelial carcinoma-associated 1 (UCA1), which is an lncRNA that contains three exons and encodes two transcripts (75), was first detected overexpression in bladder cancer (76). Its expression level is related to cellular proliferation and migration (77). The expression levels of AC026904.1 and UCA1 in metastatic breast cancer were reported to be higher than those in non-metastatic breast cancer (31).

Huang et al. proved UCA1 to be an oncogenic lncRNA in BC either *in vitro* or *in vivo*. UCA1 bound to hnRNP I (heterogeneous nuclear ribonucleoprotein I) and formed a complex which made itself more stabilized. While promoting the translational ability of p27 through hnRNP I alone, the protein content of p27 was widely suppressed when it interacted with UCA1 (78). Current research has observed that TGF-β activates UCA1 and AC026904.1 through Smad and ERK pathways, respectively. AC026904.1 activates the cis-transcription of the Slug gene in the form of eRNA. UCA1 promotes the expression of Slug in breast cancer by directly titrating miR-1 and miR-203a at the post-transcriptional level. Both of them cooperated with each other to upregulate the expression of Slug at the transcriptional and post-transcriptional levels (31). The Wnt/ β-catenin signaling pathway is recognized as a common signaling pathway related to EMT and the metastasis of breast cancer (79, 80). After transfection of si-UCA1 into MDA-MB-231 cells, the expression of negative regulators p-gsk-3 β and gsk-3 β in the Wnt signaling pathway was significantly increased. At the same time, UCAl gene knockout inhibited the protein expression of β-catenin and its downstream genes (including cyclinD1 and MMP7) (51).

TINCR

TINCR (terminal differentiation-induced non-coding RNA) is a spliced lncRNA which produces a 3.7-kb transcript. It was initially discovered from well-differentiated human somatic tissue and was proven to be an indispensable component of normal differentiation of the epidermis (81). Liu and his colleagues reported TINCR to be an oncogenic factor for BC (82); however, whether it is because of drug resistance or resistance-induced tumor metastasis remains unknown.

Compared with other sensitive cells, TINCR was critically upregulated in trastuzumab-resistant tumor cells. While the drug resistance and EMT program could be reversed through the knockdown of TINCR, Dong et al. demonstrated that TINCR induced by acetylation of H3K27 upregulates miR-125b, and promotes the EMT process by increasing the expression of Snail-1. Experimental distant metastasis in the lung was formed by injections into the lateral tail vein of mouse models. *In vivo*, compared to the sh-NC group, both the deep lung metastasis and the visible metastasis on the surface of the lung were distinctly lesser in the experimental group (32).

BORG

BMP/OP-responsive gene (BORG) in a C2C12 mouse myoblast cell line which trans-differentiates into osteoblastic cells reacts to bone morphogenetic proteins (BMPs) (83), particularly alters the proliferation of disseminated BC cells.

Alex et al. revealed that high expression of BORG is closely associated with recurrent and metastatic disease, making it a potential biomarker which is able to identify women who are at high risk of developing BC with high invasiveness or metastatic ability. In theory, BORG induces the metastatic colonies of potent BC cells by activating the transcriptional repressive activity and localization of TRIM28, which combines with BORG and leads to a great amount of changes in cancer progression. In addition, downregulated BORG in metastatic BC enhances their metastatic ability in animal models, indicating that BORG could promote alterations at both epigenetic and genetic levels, therefore underlying the development of recurrent and metastatic BC (33).

LincIN

LincIN is a long intergenic non-coding RNA between ITGB1 and NRP1 (LincIN), using a high-density SNP array-based gene expression approach to assess the lncRNA transcriptome in paired tumor tissue vs. normal samples.

Compared to adjacent normal tissue, it is frequently observed with highly expressed LincIN in tumors, and it is reported that LincIN is significantly correlated with aggressive BC. Moreover, analysis of TCGA data also supports that upregulation of LincIN is associated with poor prognosis in BC patients. Conversely, deletion of LincIN suppresses the invasion and migration of

tumor cells in experimental studies, and transcriptome analysis of the LincIN-deletion tumor cells agrees with this result. Importantly, the loss of LincIN decreases the amount of lung metastasis in tail vein injected- mice models. A study suggested that LincIN exerts a critical role in translational alterations by cooperating with NF90 to suppress the translation of p21, and the upregulation of nuclear p21 by LincIN knockdown may be related to a less aggressive phenotype in a metastasis model (34).

ANCR

An lncRNA termed anti-differentiation ncRNA (ANCR or DANCR), an 855-nucleotide lncRNA, was first discovered during the differentiation process with its downregulated expression (84).

Li and his colleagues revealed that ANCR exerts a pivotal role in BC progression and metastasis, mostly through diminishing EZH2 (enhancer of zeste homolog 2) stability. EZH2, as a pivotal inducer and regulator of EMT at the epigenetic level, contributes to multiple cancer metastases. They initially found that in contrast to normal breast tissue, the expression level of ANCR was lower both in BC cell lines and in BC samples. ANCR-mediated EZH2 degradation may play a key role in weakening the ability of initiating EMT and metastasis programs in breast cancer cells. ANCR interacts with EZH2 to promote the binding of CDK1 to EZH2 and its phosphorylation at Thr-345 and Thr-487 sites, which leads to the degradation of EZH2. Through influencing the stability of EZH2, ANCR negatively regulated the process of EMT (35).

Another study showed that ANCR, as a new downstream molecule of TGF-β, plays an important role in TGF-β1-induced EMT by reducing the expression of RUNX2. TGF-β1 induces the increasing expression of HDAC3; HDAC3 then binds to the promoter region of ANCR which inhibited the transcription of ANCR. Besides, ANCR attenuates the TGF-βsignaling pathway at least in part by inhibiting the phosphorylation of Smad2/3. In an *in vivo* study, the expression level of RUNX2 in lung tissue sections was measured, uncovering a decreasing level of RUNX2 in the lung tissue of mice models which had been injected with MDA-MB-231-ANCR cells (52).

Lnc015192

In a recent study, Huang et al. found that Adam12 is overexpressed in breast cancer cell lines, and promotes mesenchymal-epithelial transition (MET) and inhibits migration and invasiveness. The knockout of Adam12 and lnc015192 inhibits migration, invasion and EMT in breast cancer cells. It has been shown that lnc015192 could be used as the ceRNA of miR-34a to modulate Adam12, but the detailed mechanism of EMT has not been defined. In order to explore the influence of lnc015192 and Adam12 on tumor metastasis *in vivo*, either lnc015192 or Adam12-deletion 4T1 cells were injected into mice models, and the results showed that a smaller number of lung metastatic focuses were induced in sh-lnc015192-injected or sh-Adam12-injected animal models than in the control group, suggesting that the loss of lnc015192 or Adam12 suppresses the invasion, migration and metastasis ability of BC cells (36).

LINC01638

A validated lncRNA, LINC01638 (long intergenic non-protein coding RNA 1683), was reported with low expression in various normal human tissues (85) and may be associated with the EMT program of HCC cells (86).

Current research has suggested that LINC01638 is expressed at a high level in TNBC cells and tissue. LINC01638 maintains the mesenchymal characteristics of TNBC cells, containing a cancer stem cell-like state and enriched EMT signature. The knockdown of LINC01638 inhibits tumor cell proliferation and invasiveness either *in vivo* or *in vitro*. Overexpression of LINC01638 is regarded as a predictor of poor prognosis of BC patients. In theory, LINC01638 interacts with c-Myc to inhibit SPOP-mediated c-Myc degradation and ubiquitination. C-Myc promotes the expression of MTDH (metadherin) at a transcriptional level and, therefore, stimulates Twist1 expression, resulting in an EMT program.

In addition, to assess the impact of LINC01638 on BC metastasis *in vivo*, LINC01638-deletion MDA-MB-231 cells were inoculated into animal models via the tail vein. A smaller number of lung metastatic nodules were revealed from the experimental group than from the control group after several days, but restoring the expression of c-Myc diminished the impact of LINC01638 deletion on tumor metastasis *in vivo*.

These findings verified LINC01638-mediated signal transduction and emphasized the key role that LINC01638 played in TNBC development and metastasis (37).

LncRNA-HIT

In addition, lncRNA-HIT (HOXA-associated transcript induced by TGF-β) also plays a key role in TGFβ-induced EMT. E-cadherin was identified as being one of the major targets of lncRNA-HIT. The effects of lncRNA-HIT on EMT, invasion and migration in a 4T1 orthotopic mouse xenograft model were rescued by the introduction of ectopic E-cadherin. These results suggest that lncRNA-HIT can be induced by TGF-β and play a critical role in TGF-β-induced EMT (53).

LncRNAs Implicated in the Liver Metastasis of BC

NKILA

Nuclear factor-κB interacting lncRNA (NKILA) is a main suppressing checkpoint for NF-κB activation in BC. A gene at chromosome 20q13 encoded NKILA, which first exerted the antimetastatic abilities of breast cancer cells. NKILA is a novel lncRNA which was detected recently by Liu et al. (38), who attempted and successfully proposed that lncRNA NKILA suppresses the activation of NF-κB signaling through combining with the NF-κB/IκB complex to cover up the phosphorylation regions of IκB; therefore, the NF-κB/IκB complex is stabilized. Additionally, both tumor samples and *in vivo* studies validated that the downregulation of NKILA in high-grade BC promotes distant metastasis (38).

A recent study found that NF-κB plays an important role in TGF-β-induced EMT. TGF-β enhanced the expression of NKILA, thus inhibiting the overactivation of NF-κB by interacting with I-κB. The expression of NKILA controls the

switch "on and off," negatively regulating NF-κB. Additionally, the upregulation of NKILA expression notably decreased liver metastasis induced by TGF-β of a malignant tumor *in vivo*. Similar to the outcomes of animal models, the expression of NKILA was negatively associated with different EMT phenotypes in BC tissue. In conclusion, it has been shown that lncRNA NKILA can modulate the TGF-β-induced EMT of breast cancer (39).

ARNILA

Androgen receptor negatively induced lncRNA (ARNILA) is located in the cytoplasm of TNBC tissues and cells, which have been demonstrated to promote invasion, EMT and metastasis in *in vivo* and *in vitro* experiments. In addition, ARNILA was reported to function as a ceRNA for miR-204 to promote its target gene Sox4's expression, which subsequently contributed to the EMT program and induced the progression of breast cancer, thereby facilitating the invasion, EMT and metastasis of TNBC (40).

MALAT1

In a recent study, which identified MALAT1 as a target of miR-1, it was found that the expression level of miR-1 was negatively correlated with the expression of MALAT1 in BC samples. MiR-1 suppressed the development of BC, decreased cell motility and activated apoptosis by regulating MALAT1. The knockdown of MALAT1 could partly imitate the tumor-inhibitive impact of miR-1. As part of *in vivo* metastatic experiments, MCF7 cells transduced stably with miR-1 formed fewer and smaller lung and liver metastases than the control group. Such outcomes indicated that miR-1 could suppress the metastasis of BC cells by regulating MALAT1 *in vivo* (19).

lncRNAs Implicated in the Brain Metastasis of BC

lnc-BM

LncRNA associated with brain metastasis (Lnc-BM) has been demonstrated to be a prognostic factor of the progression of brain metastasis in BC patients. In preclinical experiments, upregulated expression of Lnc-BM promoted BCBM, while the knockdown of Lnc-BM with nanoparticle-encapsulated siRNAs effectively improved BCBM in murine models. In BC cells, Lnc-BM drove the STAT3-dependent expression of CCL2 and ICAM1, which acts as a mediator in the process of recruitment of macrophages and vascular co-option in the cerebrum. Macrophages which were recruited inversely produced IL-6 and oncostatin M, thereby further activating the Lnc-BM/JAK2/STAT3 pathway and promoting BCBM by mediating interaction between BC cells and the brain microenvironment (41).

XIST

X-inactive-specific transcript (XIST) is a lncRNA that participates at the beginning of X chromosome inactivation during early embryogenesis. Numerous human malignant tumors are accompanied by the deficiency of inactivated X chromosomes (Xi) as well as the duplication of active X chromosomes (87). Such a phenomenon is more common in several cancers such as breast cancer.

Here, Xing et al. found that XIST was notably decreased in BM tissue from patients with BC. The expression level of XIST was inversely associated with BM in BC patients. A loss of XIST preferentially facilitated the BM growth of XIST-high cells in xenograft animal models. Additionally, deletion of XIST in the breast of mice models enhanced the growth of BC and BM. Downregulation of XIST activated EMT and stimulated c-Met through stabilizing MSN-mediated protein, which contributed to the activation of tumor cells' stemness. Deletion of XIST also drove the secretion of exosomal miRNA-503, which consequently triggered M1–M2 polarization of microglia. However, the underlying mechanism of EMT remains unclear (42).

Others

In addition, there are some other lncRNAs which have been reported to be associated with the brain metastasis of breast cancer patients, such as NCT02915744 III, which is related to metastatic BC with brain metastases, and NCT02000882 II, which is found in TNBC patients with brain metastases (88).

LncRNAs Implicated in Other Metastasis of BC

HOTAIR

MiR-7, which was initially detected from human MDA-MB-231 and MCF-7 cell lines, was lowly expressed in breast cancer stem cells (BCSCs). Zhang and his colleagues found that MiR-7 suppressed the metastasis of BCSCs in many different organs, such as kidneys, lungs as well as adrenal glands, in NOD/SCID mice. Moreover, downregulated miR-7 may be indirectly owing to HOTAIR through regulating the expression level of HoxD10, which enhances miR-7 expression in BCSCs (43).

CONCLUSION

In the past few decades, lncRNAs have experienced a long history, from being regarded as non-functional (6) to being confirmed as an indispensable contributor to the development and progression of a variety of malignant tumors, attracting a considerable amount of attention from multiple investigators who have devoted themselves to research on lncRNAs and malignant tumors, especially tumor metastasis (89), which would directly result in cancer-related death.

In this review, we briefly illustrated those lncRNAs involved in regulating the metastatic ability of breast cancer and tried to describe the potential mechanisms, especially through the EMT process, as well as attempting to explore some novel strategies for targeting highly invasive BC. Depending on the screening results of lncRNAs, a large number of them are demonstrated to function as a promoter in the process of invasion, migration, EMT or metastasis of BC, while a small number have contradictory effects to inhibit the metastasis of BC. They regulated the distant metastatic process at different levels such as transcription levels or post-transcription levels, and it seems that there are several signaling pathways and transcription factors that are similar

among different lncRNA-involved pathways, which may be novel therapeutic targets for cancer patients in the future. However, the underlying mechanisms regarding how most of the lncRNAs regulate breast cancer remain under-defined (90).

Some studies verified whether different lncRNAs affect the distant metastasis of breast cancer through injecting the breast cancer cells with or without lncRNAs into the animal models, and observe the size and the quantity of the distant metastatic colonizations to discover the underlying mechanisms among the process of distant metastasis (28, 35, 37, 46). It has been reported that EMT mediates the dissemination of tumor cells from primary lesions, while MET is essential for the seeding of circulating tumor cells to distal organs (91–93). We intended to summarize the metastatic mechanisms to different organs accordingly, however, most of them focused on the initiation mechanism of metastasis, such as EMT, there are less studies tried to find the mechanism associated with the different metastatic organs. Huang et al. reported that lnc015192 knockdown enhanced the MET process in the lung metastasis while the underlying mechanism remains unclear (36). Another study showed that ANCR, as a new downstream molecule of TGF-β, plays an important role in TGF- β1-induced EMT by reducing the expression of RUNX2. They uncovered a reduced RUNX2 level in nude mice lung tissues injected with MDA-MB-231-ANCR cells (52). Brain metastasis has been reported with several organ-specific lncRNAs in our review, XIST expression was found significantly downregulated in brain metastatic lesions compared with other metastatic tumors based on an organ-specific cohort analysis, deletion of XIST also drove the secretion of exosomal miRNA-503, which consequently triggered M1–M2 polarization of microglia in the brain (42). Lnc-BM drove STAT3-dependent expression of CCL2and ICAM1, acting as a mediator in the process of recruitment of macrophages and vascular co-option in the cerebrum. And then promoted BCBM via activating the Lnc-BM/JAK2/STAT3 pathway (41). It seems that some lncRNAs are still related with organ-specific metastasis in breast cancer patients, but without enough studies to support an exact classification, further studies should pay more attention on this direction.

The aforementioned studies demonstrated that the lncRNAs upregulated or downregulated in BC cells or tissue are promising targets for therapy and predictors of prognosis. Nevertheless, the current problem with their clinical application is how to find a way in which to detect these lncRNAs conveniently. As we all know, lncRNAs that are usually located in the cytoplasm or nucleus of tumor cells may interact with DNA, mRNA, miRNA or protein to perform their various functions (94, 95). Nowadays, several scientists are exploring the detection of lncRNAs in the circulatory system. LncRNA HOTAIR has been detected in patients' serum with various malignant tumors and might be a potential biomarker for diagnosis and prognosis prediction of BC (96). Extracellular lncRNA-SNHG14 was capable of being equipped in exosomes and then transmitted to sensitive cells, consequently diminishing drug resistance (97). In addition, circulating PVT1 DNA increases notably in the serum of BC patients to be detected. Compared with PVT1 RNA, DNA is a more common form of the PVT1-derived segment (49). These findings totally indicate that lncRNAs-relevant factors play a critical role in BC and are possible targets for examination and therapy, which might be a more convenient method to act as a prognosticator for BC patients in the future.

AUTHOR CONTRIBUTIONS

All authors participated in designing the concept of this manuscript. YW, LW, CY, and KH reviewed the literature and drafted the article. CP, SZ, and AS finalized the paper and provided suggestions to improve it.

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MicroRNA-153 Decreases Tryptophan Catabolism and Inhibits Angiogenesis in Bladder Cancer by Targeting Indoleamine 2,3-Dioxygenase 1

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Background: Metastasis is the primary cause of cancer deaths, warranting further investigation. This study assessed microRNA-153 (miR-153) expression in bladder cancer tissues and investigated the underlying molecular mechanism of miR-153-mediated regulation of bladder cancer cells.

Methods: Paired tissue specimens from 45 bladder cancer patients were collected for qRT-PCR. The Cancer Genome Atlas (TCGA) dataset was used to identify associations of miR-153 with bladder cancer prognosis. Bladder cancer tissues and immortalized cell lines were used for the following experiments: miR-153 mimics and indoleamine 2,3-dioxygenase 1 (IDO1) siRNA transfection; Western blot, cell viability, colony formation, and Transwell analyses; nude mouse xenograft; and chicken embryo chorioallantoic membrane angiogenesis (CAM) assays. Human umbilical vein endothelial cells (HUVECs) were co-cultured with bladder cancer cells for the tube formation assay. The luciferase reporter assay was used to confirm miR-153-targeting genes.

Results: miR-153 expression was downregulated in bladder cancer tissues and cell lines, and reduced miR-153 expression was associated with advanced tumor stage and poor overall survival of patients. Moreover, miR-153 expression inhibited bladder cancer cell growth by promoting tumor cell apoptosis, migration, invasion, and endothelial mesenchymal transition (EMT) *in vitro* and tumor xenograft growth *in vivo*, while miR-153 expression suppressed HUVEC and CAM angiogenesis. At the gene level, miR-153 targeted IDO1 expression and inhibited bladder cancer cell tryptophan metabolism through inhibiting IL6/STAT3/VEGF signaling.

Conclusions: Collectively, our data demonstrate that miR-153 exerts anti-tumor activity in bladder cancer by targeting IDO1 expression. Future studies will investigate miR-153 as a novel therapeutic target for bladder cancer patients.

Keywords: bladder cancer, miR-153, tryptophan catabolism, angiogenesis, indoleamine 2, 3-dioxygenase 1

INTRODUCTION

Bladder cancer is one of the most commonly occurring malignancies worldwide, accounting for an estimated 429,800 new cases and more than 165,000 cancer-related deaths in 2012, with an increased prevalence in males (1). Risk factors of bladder cancer include tobacco smoke, family cancer history, frequent bladder infections, and exposure to certain chemicals (2, 3). To date, ∼75% of clinically diagnosed bladder cancers are non-muscle invasive and 25% involve bladder muscle (4). Treatment options for bladder cancer depend on tumor stage and include transurethral resection (TURBt), intravesical instillation, immunotherapy [Bacillus Calmette–Guérin (BCG)], and chemotherapy (5, 6). Compared to non-muscle invasive bladder cancer, tumors invading the muscle often have a poor prognosis (4). However, high rates of tumor recurrence are a clinical burden for all bladder cancer patients. Although we have obtained some useful results in study of the molecular mechanisms of bladder cancer (7, 8), further studies of bladder cancer etiology, molecular mechanisms, and pathogenesis could help identify novel strategies for early diagnosis and effective control of bladder cancer.

MicroRNA (miRNA or miR) is a class of single-stranded, non-coding RNA molecules that are ∼18–22 nucleotides in length (9). MiRNAs function post-translationally to regulate expression of protein-coding genes through binding to the untranslated region (UTR; most at the 3′ end) of target mRNAs to promote either degradation of the targeting mRNAs, suppression of mRNA translation, or both. Thus, miRNAs negatively regulate the effect of targeting genes in cells (10). miRNAs can regulate cell growth, differentiation, migration, cell cycle, apoptosis, angiogenesis, and embryo development, whereas alteration of miRNAs could induce development of human diseases and carcinogenesis (9). To date, many studies have shown aberrant miRNA expression in human cancers, including bladder cancer (11–13). For example, a recent study revealed a urinary microRNA signature for accurate diagnosis of bladder cancer, independent of tumor stage and grade (14). Another recent study comprehensively analyzed the miRNA-mRNA regulatory network in gemcitabine-resistant bladder cancer cells (15), while a recent integrated analysis confirmed the usefulness of miRNAs as novel predictors of urologic cancer prognosis (16). Pardini et al. profiled different miRNA expression patterns to stratify bladder cancer subtypes (17). Moreover, Fujii et al. reviewed recent advancements in miRNA research with regards to smoking-related carcinogenesis to develop novel biomarkers and therapeutic strategies and better understand miRNA functions in cells (18). Robertson et al. performed a comprehensive analysis of 412 muscle-invasive bladder cancers using multiple TCGA datasets to identify expression of various mRNAs, long non-coding RNAs, and miRNAs for differential epithelial-mesenchymal transition (EMT) status of bladder cancer, carcinoma *in situ*, histology, and prognosis, as well as response of patients to different treatments (19). van Kampen et al. revealed the effects of a given miRNA to reverse tumor cell EMT and gene targeting (20).

miR-153 was reported to play an important role in the development of several types of human cancers. For example, a recent study showed that chromatin-modifying drugs upregulated miRNA-153 expression in glioblastoma cell lines, indicating that miR-153 is a tumor suppressor gene (21). miR-153 was downregulated in osteosarcoma tissues and expression of miR-153 suppressed osteosarcoma cell proliferation and invasion *in vitro* (22). miR-153 was also shown to be predictive of gastric cancer prognosis, and expression of miR-153 inhibited tumor cell migration and invasion capacity (23). Thus, in this study, we first assessed miR-153 expression in bladder cancer compared to adjacent normal tissue specimens and then investigated the underlying molecular mechanism of miR-153 mediated bladder cancer cell regulation. Our study provides new information regarding miR-153 as a potential tumor biomarker or novel therapeutic target for bladder cancer.

MATERIALS AND METHODS

Patients and Tissue Specimens

This study collected normal and cancerous tissue specimens from 45 bladder cancer patients from Shanghai Tenth People's Hospital, Tongji University (Shanghai, China) between January 2017 and January 2018. These patients were histologically diagnosed with bladder cancer and did not receive any intravesical instillation or chemotherapy before surgery. Fresh tissue specimens were harvested during surgery and immediately snap-frozen in liquid nitrogen and stored at −80°C until use. This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital and each patient provided informed consent before enrolling in the study. Clinicopathological data were retrieved from medical records, as shown in **Table 1**.

TABLE 1 | Association of miR-153 expression with clinicopathological data from patients.

Parameters	No. of patients	High expression	Low expression	*P*-value
Gender				1.00
Male	42	7	35	
Female	3	1	2	
Age (years)				0.39
≤65	19	4	15	
>65	26	4	22	
Histologic grade				1.00
High	41	7	34	
Low	4	1	3	
Tumor stage				0.031
I-II	20	6	11	
III-IV	25	2	26	
Tumor size (cm)				0.030
>3	23	2	27	
≤3	22	6	10	
Tumor Progress				0.009
Yes	27	1	26	
No	18	6	12	

Reagents and Elisa Assay

Human recombinant interleukin 6 (IL-6; PeproTech, USA) was dissolved in trehalose at a concentration of 100 μg/mL and stored at −20°C. At the time of use, the final concentration of IL-6 was adjusted to 100 ng/mL in the appropriate medium. Human IL6 Elisa kit was purchased from Anogen-Yes Biotech Laboratories Ltd (EL10023, Ontario, Canada) and the concentration of IL6 in the cell supernatant was determined according to the manufacturer's instructions.

Retrieval of the Cancer Genome Atlas (TCGA) Dataset

The Cancer Genome Atlas (TCGA) is a web-based database that attempts to map genomic variants of selected human cancers through large-scale genome sequencing of miRNA arrays and RNA-Seq. Currently, the TCGA contains 50 tumors and their subtypes. In this study, we downloaded the bladder cancer dataset (http://tcga-data.nci.nih.gov/tcga/) and assessed miR153 expression in 408 bladder cancer tissues and 19 normal tissues.

Cell Lines, Culture, and Transfection

Human bladder cancer T24, UMUC3, 5637, and J82 cell lines and an immortalized human normal bladder epithelial cell line, SV-HUC-1, were obtained from the Chinese Academy of Sciences (Shanghai, China). T24, UMUC3, and 5637 cell lines were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), J82 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.), and SV-HUC-1 cells were grown in F12K medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) in a humidified incubator containing 5% CO_2 at 37°C.

To overexpress miR-153 in tumor cells, we purchased miR-153 mimics and the negative control from Genechem (Shanghai, China) into bladder cancer cell lines using.T24 and UMUC3 cells were seeded into 6-well plates in RPMI-1640 media supplemented with 10% FBS. At 70% confluence, the cells were transfected with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were harvested 48 h after transfection and subjected to analysis by qRT-PCR or western blotting. To knockdown IDO1 expression in tumor cells, we obtained IDO1 siRNA and negative control siRNA from Genepharma (Shanghai, China). We transfected the siRNAs into bladder cancer cell lines using Lipofectamine 3000 (Invitrogen). These transfected cell lines were then subjected to different assays. These sequences are shown in **Supplementary Table 1.**

RNA Isolation and qRT-PCR

Total cellular RNA was isolated from tissue specimens and cell lines using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using a cDNA synthesis kit (Takara, Dalian, China) according to the manufacturers' instructions. qPCR amplification was done using the SYBR Green PCR Kit (Takara) in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers were show in **Supplementary Table 1**. U6 was used as the internal control for miR-153 mimics, and GAPDH was used as the internal control for IDO1. The PCR parameters were set for an initial cycle of 95°C for 2 min and 40 cycles at 95°C for 30 s, 57°C for 45 s, and 72°C for 45 s. The relative levels of each gene were analyzed using the $2^{-\Delta\Delta Cq}$ method. Experiments were done in duplicate and repeated three times.

Western Blot

Total cellular protein was extracted from cells or tissues using radio immunoprecipitation assay buffer (RIPA buffer; Beyotime, Shanghai, China) and quantified.

The reaction mixture was incubated on ice for 30 min and centrifuged for 10 min at 12,000 g at 4°C. The supernatant was collected, and the protein concentration was estimated using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.) Proteins (30 μg per sample) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gels and transferred onto nitrocellulose (NC) membranes (Sigma-Aldrich). The membranes were blocked in 5% skim-milk solution in phosphate buffered saline (PBS) for 1 h at room temperature and then immunoblotted with a primary antibody at 4°C overnight. Primary antibodies included: IDO1 (1:1,000, ab211017, Abcam, USA), STAT3 (1:1,000, ab68153, Abcam), phosphorylated (p)-STAT3 (1:1,000, ab76315, Abcam), vascular endothelial growth factor (VEGF) (1:1,000, ab52917), E-cadherin (1:5,000, ab40772, Abcam), N-cadherin (1:5,000, ab76011, Abcam), and vimentin (1:5,000, ab92547, Abcam). β-actin (sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control. On the next day, the membranes were washed with PBS-Tween 20 (PBS-T) thrice and then incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature and then visualized with the Odyssey two-color infrared laser imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Cell Viability and Colony Formation Assays

Cell viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). In brief, transfected cells were seeded into 96-well plates at a density of 1×10^3 cells/well and cultured for up to 3 days. After 6, 24, 36, or 72 h, we added 10 μl of the CCK-8 reagent into each well and further cultured the cells at 37°C for 2 h and then measured the absorbance rate on a microplate spectrophotometer (BioTek, Instruments, Inc., Winooski, VT, USA) at 450 nm.

For the cell colony formation assay, transfected cells were seeded in 6-well plates at 1×10^3 /well and grown for 14 days with regular cell growth medium refreshment. At the end of each experiment, cells were washed three times with ice-cold PBS, fixed with 75% ethanol, and then stained with 0.1% crystal violet solution. Tumor cell colonies were counted from cell photographs that were imaged using an inverted microscope with a digital camera (Olympus Corporation, Tokyo, Japan).

Tumor Cell Transwell Migration and Invasion Assay

Transwell chambers were obtained from Corning (Lowell, MA, USA). For the assay, 5×10^4 transfected cells were seeded into the upper chamber of the Transwell in 200 μl medium without

FCS, while the bottom chamber was filled with 600 μl culture medium containing 10% FCS. Cells were cultured for 16 h for the tumor cell migration assay or 24 h for invasion assay at 37°C. Cells that remained in the top surface of the Transwell filters were removed using a cotton swab, and the cells that migrated into or invaded the low surface of the filters were washed three times with iced-cold PBS, fixed with 70% ethanol for 30 min, stained with 0.5% crystal violet solution for 15 min, and counted under a light microscope (Olympus Corporation, Tokyo, Japan). For the cell invasion assay, the Transwell filters were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Nude Mouse Xenograft Assay

The animal protocol in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Tenth People's Hospital, Tongji University (Shanghai, China) and followed the regulatory animal care guidelines of the United State National Institute of Health (Bethesda, MD, USA). In brief, eight 4–5 week old male nude BALB/c mice were purchased from Shanghai SIPPR-Bk Lab Animal Co., Ltd. (Shanghai, China) and maintained in a specific pathogen-free (SPF) "barrier" facility and housed under controlled temperature and humidity with alternating 12 h light and dark cycles. Mice received SPF mouse chow and were allowed to drink sterile water *ad libitum*. Mice were subcutaneously injected with 1 x 10^6 cells and tumor xenograft volume and size were measured weekly with a vernier caliper and calculated as follows: length × width^2/2. After 40 days, we sacrificed the mice and collected all tumor xenografts for analysis.

Tube Formation Assay

The capillary tube formation assay was used to assess the effect of miR-153 on HUVEC angiogenesis *in vitro*. In brief, Matrigel (12.5 mg/ml from BD Biosciences, Bedford, MA, USA) was thawed overnight at 4°C and 50 μl Matrigel was then added into each well of a 96-well plate and allowed to solidify at 37°C for 1 h. Then, 5,000 HUVECs were plated into each well and cultured in medium containing supernatants of miR-153 or control cultured cells for 6 h. Tube formation was observed under an inverted microscope (Olympus Corporation, Tokyo, Japan), photographed, counted, and summarized.

Chicken Chorioallantoic Membrane (CAM) Assay

SPF fertilized eggs (Health-Tech Lab, Shandong, China) were incubated at 37°C for 5 days under constant humidity and a window on the shell was opened to expose the CAM. Next, 5 × 10^6 cells were added onto the CAM and the eggs were incubated for 48 h to assess vascular branches and lengths, which

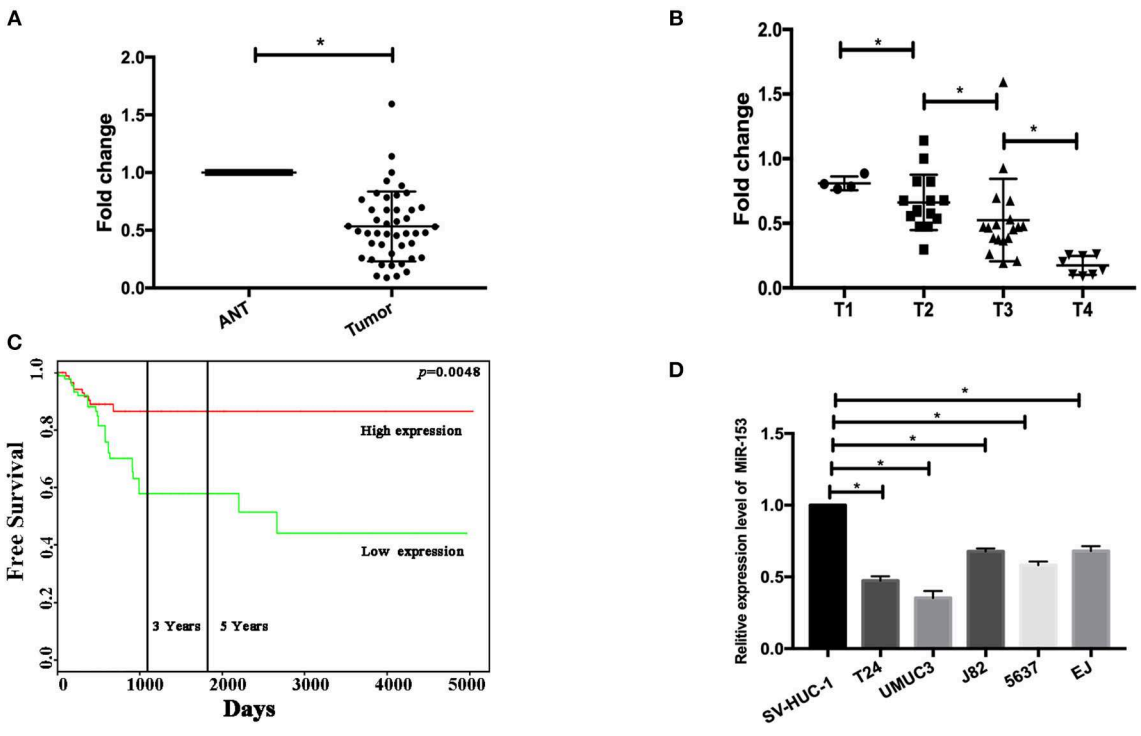

FIGURE 1 | miR-153 is downregulated in bladder cancer tissues and cell lines. **(A)** qRT-PCR. miR-153 was detected in 45 pairs of bladder cancer (tumor) and adjacent normal tissues using qPCR ($P < 0.05$). **(B)** Association of miR-153 with clinicopathological features. Decreased miR-153 levels are associated with advanced tumor T stages. **(C)** Kaplan-Meier curve and log rank test stratified by miR-153 expression in TCGA dataset (http://tcga-data.nci.nih.gov/tcga/). **(D)** qRT-PCR. Various bladder cancer cell lines (T24, UMUC3, J82, 5637, and EJ) and an immortalized bladder epithelial cell line (SV-HUC-1) were grown and used for qPCR analysis (*$P < 0.05$).

were quantified using angiogenesis measurement software from KURABO (Osaka, Japan).

Flow Cytometry

Apoptosis was measured using the Annexin V-FITC Apoptosis Kit (BD Biosciences, Erembodegem, Belgium). Transfected cells were harvested, washed twice with cold PBS and resuspended in Annexin V binding buffer. Thereafter, the cells were stained with fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min at 4° C in the dark. Apoptosis rate was measured using a BD FACS Calibur (Beckman Coulter, CA, USA).

CD34 expression on HUVECs was assayed using flow cytometry after co-culturing HUVECs with transfected bladder cells. In brief, after cell co-culture, HUVECs were collected and incubated on ice for 30 min in a fluorescence-activated cell-sorting buffer (BD Biosciences, Erembodegem, Belgium) containing a mouse anti-human CD34 antibody (#550761; BD Biosciences) and then analyzed using BD FACS Calibur. The percentage of positive cells was quantified using FlowJo software (BD Biosciences). The experiments were performed in duplicate and repeated three times.

Dual Luciferase Reporter Assay

The target genes were predicted using bioinformatics analysis tools, including TargetScan (http://www.targetscan.org/vert_72/), ComiR (http://www.benoslab.pitt.edu/comir/) and miRANDA (http://www.microrna.org/).

To confirm the presence of miR-153 binding sites in the IDO1 3′-UTR, IDO1-wild-type 3′-UTR (IDO1-wt), and IDO1-mutant 3′-UTR (IDO1-mut) luciferase pmirGLO reporter vectors were constructed. Specifically, cells were seeded at a density of 1×10^5 per well into 12-well plates and grown overnight and then co-transfected with either 100 nmol of the miR-153p mimics or the miR-NC plus 100 ng pmirGLO-30-UTR plasmid using Lipofectamine 3000 (Invitrogen) for 48 h. Next, total cellular protein was extracted and quantified using a routine laboratory protocol. Luciferase activity was measured using a dual luciferase reporter system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) Analysis

Cells were seeded into 10 cm culture dishes and cultured for 24 h and then centrifuged at 10,000 g for 10 min at 4°C to collect the supernatant for metabolomics analysis. Cell supernatants were separated using the Agilent 1290 Infinity LC Ultra High-Performance Liquid Chromatography System (UHPLC) HILIC column at 25°C. The flow rate was 0.3 mL/min following

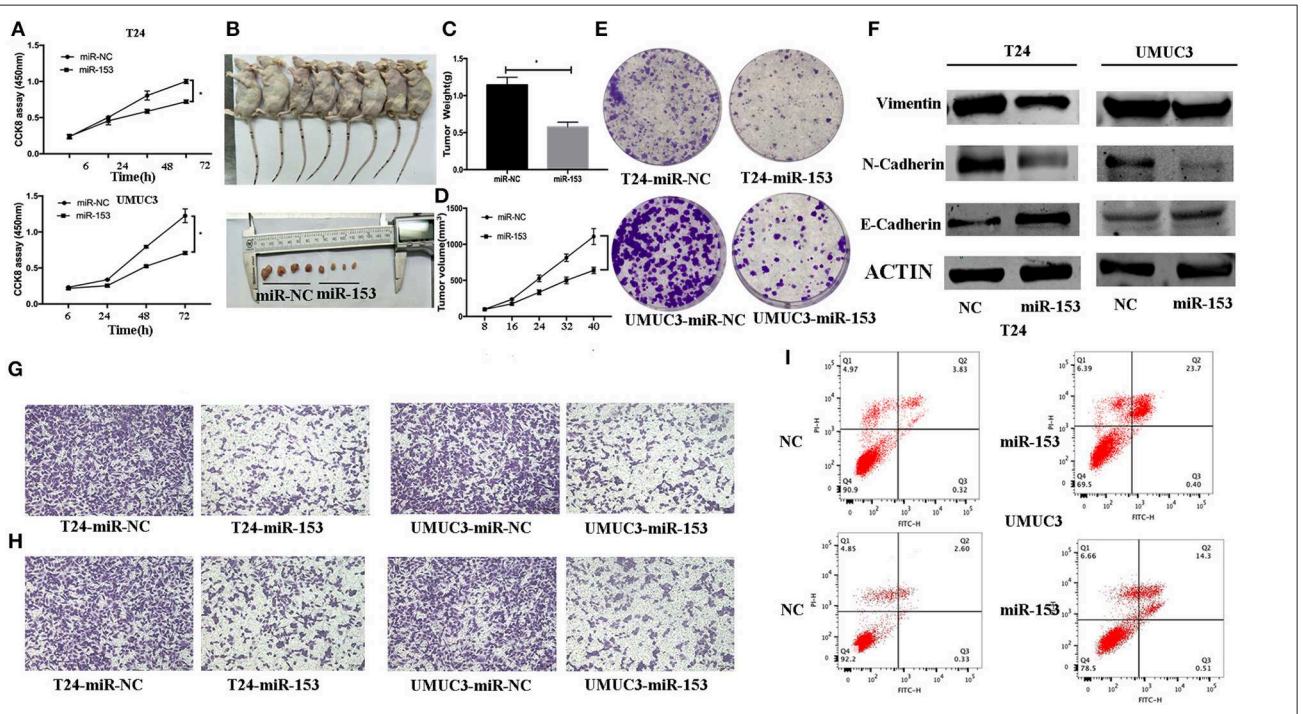

FIGURE 2 | miR-153 inhibits bladder cancer growth *in vitro* and *in vivo* by promoting tumor cell apoptosis, migration, invasion, and EMT. **(A)** Cell viability CCK-8 assay. T24 and UMUC3 cells were transfected with miR-153 mimics or negative control and then subjected to the CCK-8 assay. **(B)** Nude mouse xenograft assay. Stably miR-153 expressing mimics or negative control bladder cancer cells were subcutaneously injected into nude mice and monitored for 40 days for tumor cell xenograft formation and growth. **(C)** Tumor cell xenograft growth curves. **(D)** Tumor cell xenograft weight. **(E)** Colony formation assay. T24 and UMUC3 cells were transfected with miR-153 mimics or negative control and then subjected to tumor cell colony formation assay (x 200). **(F)** Western blot. Expression levels of EMT-associated markers in miR-153 mimics or negative control transfected T24 and UMUC3 cells were evaluated by using Western blot analysis. **(G)** Transwell tumor cell migration assay. **(H)** Transwell tumor cell invasion assay. **(I)** Flow cytometric Annexin V-PI double staining assay. $^*P < 0.05$.

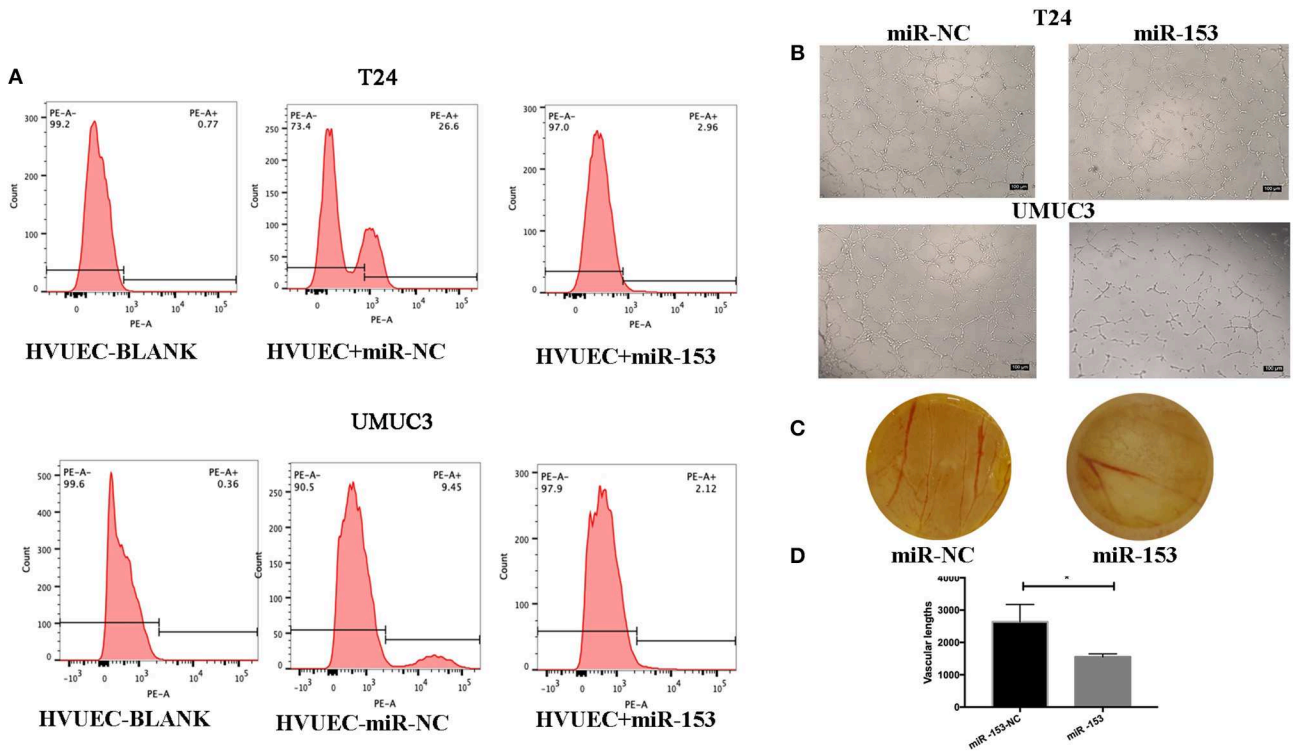

FIGURE 3 | miR-153 inhibits bladder cancer angiogenesis *in vitro* and *in vivo*. **(A)** Flow cytometric assay. CD34 expression on HVUECs was assessed using flow cytometry in after co-culture with miR-153 or negative control transfected T24 and UMUC3 cells. **(B)** HUVEC tube formation assay. HUVECs were co-cultured with T24 and UMUC3 cells using Transwells and then subjected to the capillary-like tube formation assay (x100). **(C)** The CAM angiogenesis assay. miR-153 or negative control transfected T24 cells were added on to CAM to induce angiogenesis for 48 h to induce formation of vascular branches (x 10). **(D)** The vascular length are measured (*$p < 0.05$).

an initial injection volume of 2 μL of each of sample. The conditions of the mobile phase included composition A with water, 25 mM ammonium acetate, plus 25 mM ammonia, while composition B included acetonitrile. The gradient elution was set at 0–1 min, 95% B; 1–14 min, B varied linearly from 95 to 65%; 14–16 min, B was changed linearly from 65 to 40%; 16–18 min, B was maintained at 40%; 18–18.1 min, B was changed linearly from 40 to 95%; and 18.1–23 min, B remained at 95%. The samples were then subjected to mass spectrometry using a Triple TOF 5600 mass spectrometer (AB SCIEX). Electrospray ionization (ESI) positive and negative ion modes were used for signaling detections according to the manufacturer's protocol.

Statistical Analysis

All statistical analyses were performed using the SPSS software (version 22; SPSS, Inc., Chicago, IL, USA) or Graphpad (Version 5.0, GraphPad Prism Software Inc., San Diego, CA, USA). The continuous correction Chi-square test was used to assess the association between clinicopathological features and miR-153 expression, and the Student's *t*-test was performed to analyze differences between groups. A $P < 0.05$ was considered statistically significant.

RESULTS

miR-153 Is Downregulated in Bladder Cancer Tissues and Cell Lines

In this study, we first measured miR-153 expression in bladder cancer tissue specimens and cell lines compared to normal samples and found that miR-153 expression was significantly downregulated in 45 pairs of bladder cancer compared to adjacent normal tissues (ANT; $P < 0.05$; **Figure 1A**). The reduced miR-153 expression was associated with advanced tumor stage (**Figure 1B**). However, since our patients were enrolled between 2017 and 2018 with a very short follow-up period, we used the TCGA dataset to determine the association of miR-153 levels with bladder cancer prognosis. Our Kaplan-Meier curves and log rank test showed that reduced miR-153 expression was associated with worse overall survival of patients (**Figure 1C**). Consistently, miR-153 expression was also lower in different bladder cancer cell lines T24, UMUC3, 5637, and J82, compared to expression in the normal bladder epithelial cell line SV-HUC-1. Among these tumor cell lines, T24 and UMUC3 cells expressed the lowest level of miR-153 (**Figure 1D**) and were therefore used for overexpression of miR-153 in the following experiments.

miR-153 Inhibits Bladder Cancer Growth *in vitro* and *in vivo* by Promoting Tumor Cell Apoptosis

Next, we assessed the effects of miR-153 expression on bladder cancer cell proliferation. Transfection of miR-153 mimics into T24 and UMUC3 cells significantly increased miR-153 expression compared to miR-NC control cells (**Figure 2A**). We measured the effect of miR-1543 overexpression on tumor cell growth and found that miR-153 overexpression significantly reduced viability and colony formation in T24 and UMUC3 cells (**Figures 2A,E**). Consistently, our nude mouse experiments revealed that growth of tumor cell xenografts after miR-153 overexpression was also suppressed compared to the miR-NC groups (**Figures 2B–D**). Furthermore, the apoptosis rate of miR-153-expressing tumor cells was upregulated compared to the negative controls (**Figure 2I**).

miR-153 Inhibits Bladder Cancer Cell Migration, Invasion, and EMT

We evaluated the effect of miR-153 on bladder cancer cell migration and invasion capacity. Our data showed that miR-153 mimics significantly reduced T24 and UMUC3 cell migration and invasion compared to the negative controls (**Figures 2G,H**). Furthermore, during tumor cell EMT, E-cadherin expression (epithelial cell marker) was reduced, whereas N-cadherin and vimentin expression (mesenchymal cell markers)were increased induced (24). Our *in vitro* data showed that miR-153 overexpression reduced N-cadherin and vimentin expression, but increased E-cadherin expression (**Figure 2F**).

miR-153 Inhibition of Bladder Cancer Angiogenesis *in vivo* and *in vitro*

We also assessed the effect of miR-153 expression on tumor angiogenesis *in vitro*. We found that miR-153 overexpression altered bladder cancer angiogenesis. Specifically, co-culture of stably miR-153 expressing bladder cancer cells with HVUECs using Transwell chambers (0.4 μm) reduced the ability of HVUECs to form tube-like structures *in vitro* (**Figure 3B**). At the protein level, CD34 expression on HVUECs was significantly reduced after co-culturing with miR-153-expressing bladder cancer cells compared to control HVUECs (**Figure 3A**). Furthermore, the CAM angiogenesis assay confirmed the tube formation data, showing that miR-153 expression reduced *in vivo* angiogenesis (**Figures 3C,D**).

miR-153 Targets IDO1 Expression and Inhibits Bladder Cancer Cell Tryptophan Metabolism

Thus far, we revealed the anti-tumor activity of miR-153 in bladder cancer cells *in vitro* and *in vivo*; however, the biological

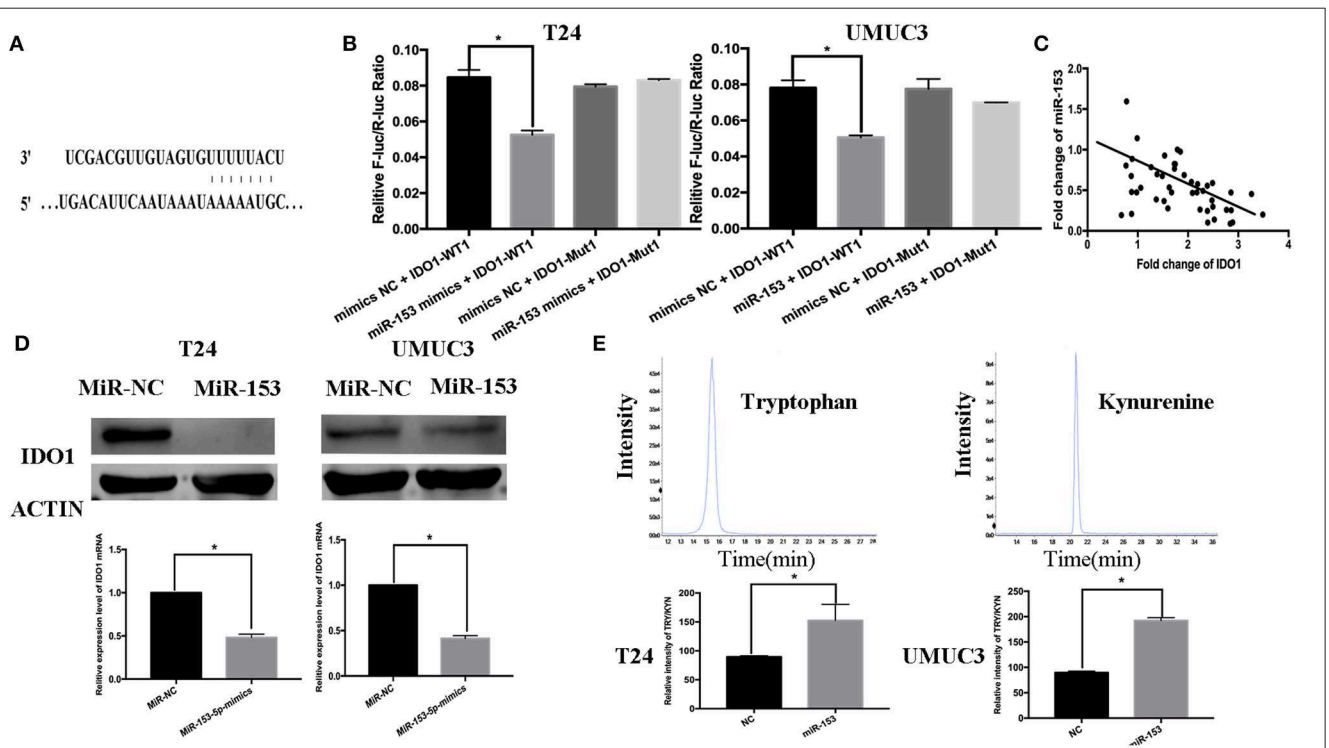

FIGURE 4 | IDO1 is a direct miR-153 target. **(A)** Bioinformatical analysis predicted that IDO1 is a direct miR-153 target. **(B)** Luciferase reporter assay. miR-153 or miR-NC-transfected bladder cancer cells were co-transfected with a luciferase reporter plasmid (WT or MUT 3 -UTR IDO1 cDNA) and then subjected to protein extraction and luciferase reporter assay. **(C)** miR-153 is negatively correlated with the expression of IDO1 in cancer tissues by qRT-PCR **(D)** qRT-PCR and Western blot. miR-153 or negative control transfected T24 and UMUC3 cells were grown and subjected to qRT-PCR and Western blot. **(E)** UHPLC-MS. miR-153 or negative control transfected T24 and UMUC3 cells were grown and the supernatants were subjected to UHPLC-MS; comparison of the tryptophan ratio to kynurenine. $^*P < 0.05$.

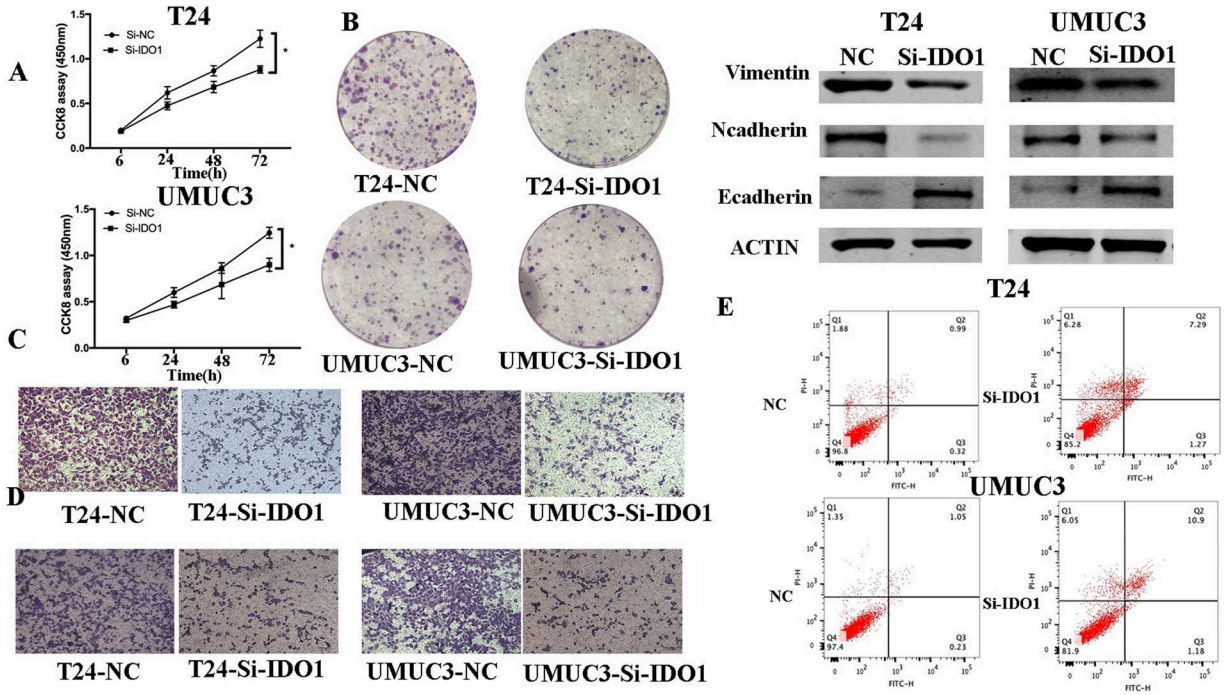

FIGURE 5 | IDO1 knockdown inhibits bladder cancer cell proliferation, migration, and invasion, and induced apoptosis and modulation of EMT markers. **(A)** Cell viability CCK-8assay. T24 and UMUC3 cells were transfected with IDO1 or negative control siRNA and then subjected to the CCK-8 assay. **(B)** Colony formation assay. T24 and UMUC3 cells were transfected with IDO1 or negative control siRNA and then subjected to colony formation (x 200) and Transwell assays. **(C)** Western blot. Levels of the EMT-associated markers were analyzed in T24 and UMUC3 cells after IDO1 knockdown by using Western blot. **(D)** Transwell migration assay. **(E)** Transwell invasion assay. **(E)** Flow cytometric Annexin V-PI double staining assay in T24 and UMUC3 cells after knockdown of IDO1. $^*P < 0.05$.

function of miRNAs is to regulate expression of their targeting genes. In this regard, we performed bioinformatical analysis to predict the target genes of miR-153. We found that IDO1 was a miR-153 target. Specifically, miR-153 is predicted to bind to the 3′-untranslated region (UTR) of IDO1 cDNA (**Figure 4A**). To confirm this, we performed a luciferase reporter assay and found that miR-153 inhibited luciferase activity of the wild type 3′-UTR IDO1 cDNA, but not of the mutated cDNA (**Figure 4B**). Furthermore, we found an inverse association of IDO1 expression with miR-153 expression in 45 bladder cancer tissue samples (**Figure 4C**). In addition, miR-153 overexpression significantly downregulated levels of IDO1 mRNA and protein in T24 and UMUC3 cells (**Figure 4D**). IDO1 is the key enzyme in tryptophan metabolism and we therefore assessed whether tryptophan metabolism was altered using UHPLC-MS of the supernatants of miR-153-expressing and negative control T24 and UMUC3 cells (**Figure 4E**). We found that miR-153 expression suppressed tryptophan metabolism, and the ratio of tryptophan/kynurenine (TRY/KYN) was significantly higher in miR-153 expressing tumor cells compared to the control cells.

IDO1 Suppression and miR-153 Overexpression Have Parallel Effects in Bladder Cancer Cells *in vitro*

To further confirm the biological function of IDO1 in bladder cancer cells, we knocked down IDO1 expression in T24 and UMUC3 cells using si-IDO1 and found that si-IDO1 significantly reduced levels of IDO1 mRNA and protein in T24 and UMUC3 cells compared to the negative controls. Knockdown of IDO1 expression reduced tumor cell growth, colony formation, migration, and invasion, but induced tumor cell apoptosis. Inhibition of IDO1 expression also modulated expression of EMT markers (**Figure 5**) and HUVEC angiogenesis. These findings are consistent with miR-153 overexpression in bladder cancer cells, suggesting that the effects of miR-153 on bladder cancer cells are mediated through targeting IDO1 expression (**Figure 6**).

miR-153 Targets IDO1 and Modulates Angiogenesis Through IL6/STAT3/VEGF Signaling

To explore the underlying molecular mechanism of miR-153 in bladder cancer, we measured interleukin-6 (IL6) expression in supernatants of miR-153-overexpressing or IDO1 knocked down bladder cancer cells compared to the negative control cells. We found that, IL-6 expression was significantly suppressed in the experimental groups compared to the control groups, while phosphorylated STAT3 and VEGF were also significantly decreased in miR-153-overexpressing or IDO1 knocked down bladder cancer cells (**Figure 7**). However, addition of exogenous IL-6 reversed levels of both phosphorylated STAT3 and VEGF expression (**Figure 7**). Thus, we speculate that the role of miR-153 in bladder cancer angiogenesis

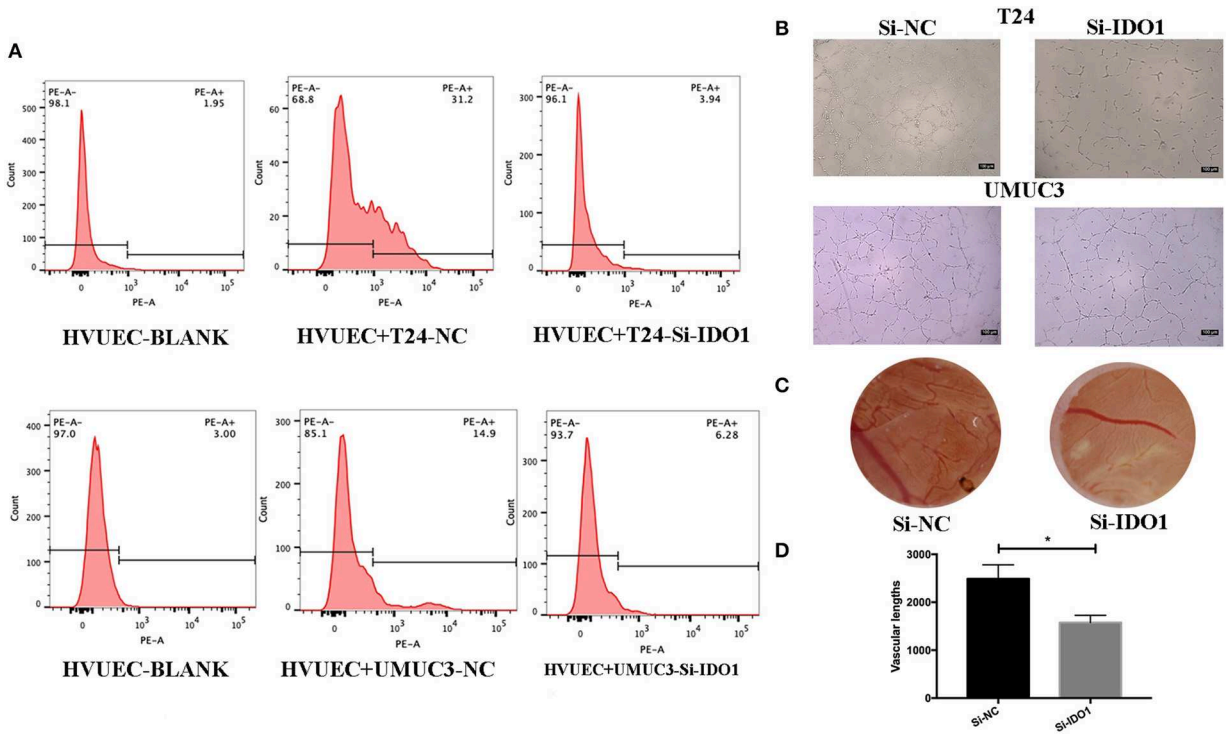

FIGURE 6 | IDO1 knockdown inhibits angiogenesis. **(A)** Flow cytometric assay. HUVECs were co-cultured with IDO-siRNA or negative control siRNA-transfected T24 and UMUC3 cells and then subjected to flow cytometric analysis of CD34 level on HUVECs. **(B)** HUVEC tube formation assay. The same cultured HUVECs were subjected to the tube formation assay (x100). **(C,D)** The CAM assays. The siRNA-transfected T24 cells were added onto CAM to modulate angiogenesis for 48 h (x 10). The branches and length are measured (*$p < 0.05$).

is to target IDO1 expression and regulate IL6/STAT3/VEGF signaling (**Figure 8**).

DISCUSSION

The role of miRNAs in cancer development and progression has recently received increasing attention. In bladder cancer, different miRNAs have been shown to be altered and associated with tumor development and progression. These miRNAs could be useful biomarkers for early detection, prediction of treatment response, subtyping, and prognosis of bladder cancer. In our current study, we measured miR-153 expression in bladder cancer compared to adjacent normal tissue specimens and then investigated the underlying molecular mechanisms of miR-153 on regulating bladder cancer cells. We found that miR-153 expression was reduced in bladder cancer tissues and cell lines, which was associated with advanced tumor stage and poor overall survival. Restoring miR-153 expression in bladder cancer cell lines inhibited tumor cell growth, migration, invasion, and EMT *in vitro* and tumor xenograft growth *in vivo*, while miR-153 expression also suppressed HUVEC and CAM angiogenesis. miR-153 targeted IDO1 expression and inhibited bladder cancer cell tryptophan metabolism through inactivating the IL6/STAT3/VEGF signaling pathway. In conclusion, our current study demonstrates that miR-153 exerts its anti-tumor activity in bladder cancer by targeting IDO1 expression.

Altered miR-153 expression and anti-tumor activity of miR-153 in bladder cancer have not been previously reported and are therefore novel findings. However, the role of miR-153 has been described in other human cancers. Xu reported miRNA-153 tumor suppressor activity in glioblastoma (21), while miR-153 was downregulated in osteosarcoma tissues (22), and loss of miR-153 expression was associated with poor gastric cancer prognosis (23). Moreover, miR-153 was downregulated in lung cancer and miR-153 expression inhibited lung cancer cell proliferation and migration, but promoted tumor cell apoptosis (25). Our current study further supports these published findings in human malignancies. However, Zhang et al. showed that miR-153 was highly expressed in advanced colorectal cancer and miR-153 promoted colorectal cancer cell invasion and cisplatin-resistance (26). Wu et al. reported that miR-153 was up-regulated in prostate cancer and miR-153 expression induced tumor cell proliferation and migration (27). These studies suggest that the role of miR-153 differs depending on the type and location of the cancer. Thus, further study of miR-153 in bladder cancer is needed to confirm miR-153's anti-tumor activity.

In our current study, we also demonstrated that the effect of miR-153 on inhibiting bladder cancer-induced HUVEC angiogenesis *in vitro* and *in vivo*. At the molecular level, miR-153 suppressed IDO1 expression by targeting IDO1 3′-UTR. Indeed, IDO1 is a rate-limiting enzyme in tryptophan metabolism (28).

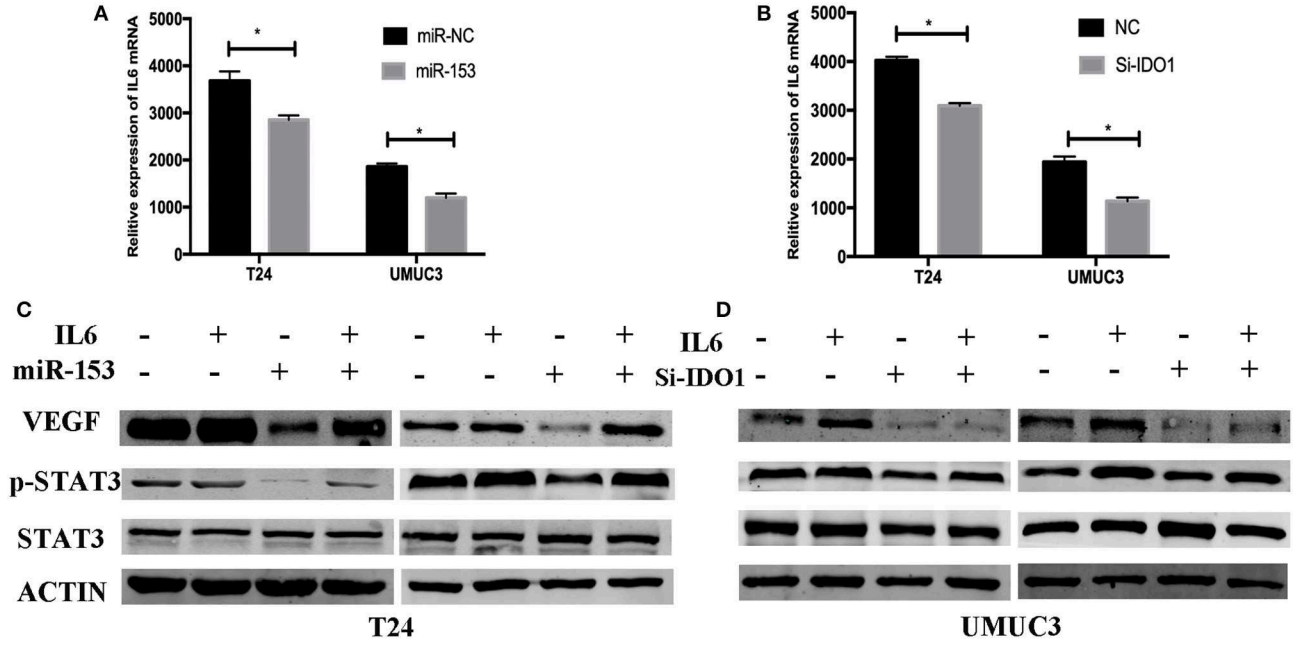

FIGURE 7 | miR-153 targets IDO1 and modulates angiogenesis through IL6/STAT3/VEGF signaling. **(A,B)** ELISA. IL_6 expression in T24 and UMUC3 cells (overexpression of miR-153 or knockdown of IDO1 and their respective negative controls) were analyzed using ELISA. **(C,D)** Western blot. T24 and UMUC3 cells (overexpression of miR-153 or knockdown of IDO1 and their respective negative controls) were pretreated with 100 ng/ml of IL-6 for 48 h and then subjected to Western blot analysis of STAT3, p-STAT3, and VEGF.

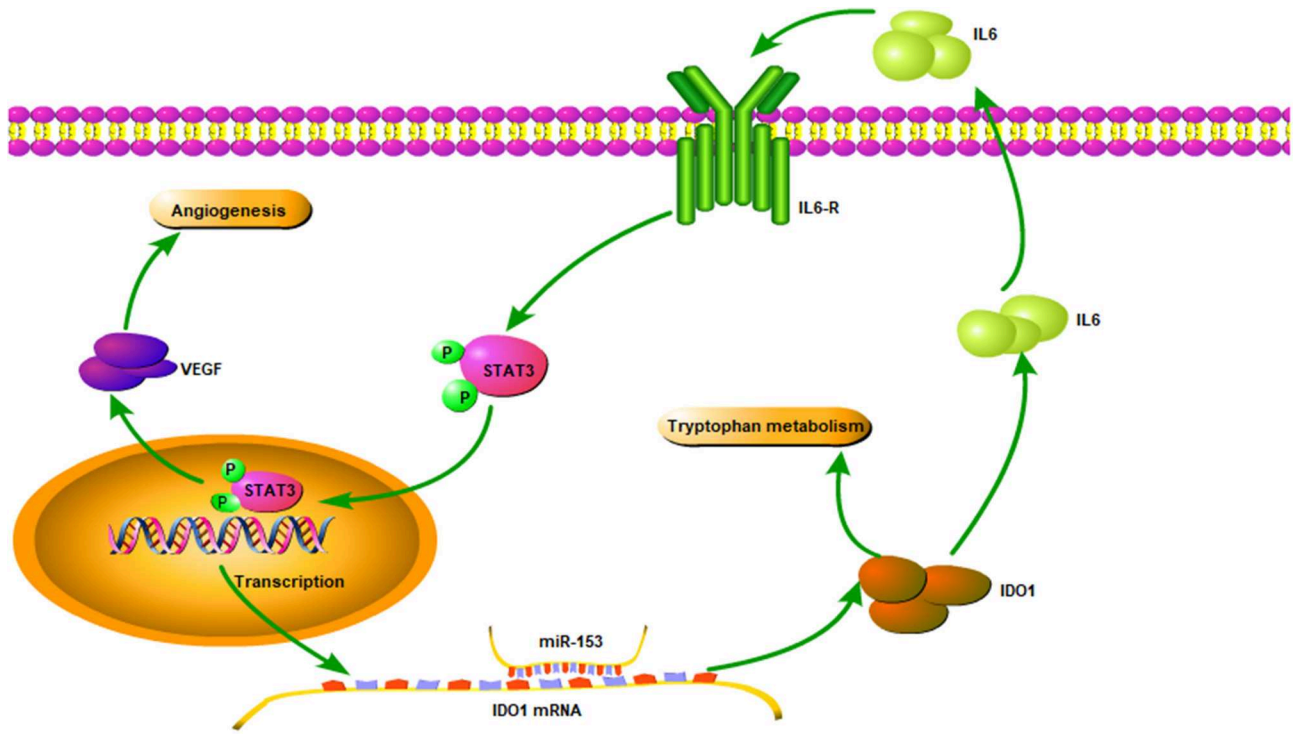

FIGURE 8 | Illustration of the miR-153 gene pathway in bladder cancer cells. miR-153 expression is lost in bladder cancer, which decreases IDO1 expression and subsequent activity of the IL6/STAT3/VEGF signaling pathway in bladder cancer cells.

The tryptophan catabolic pathway plays an important role in tumor cell escape (29, 30). Moreover, IDO1 has non-immune functions, such as angiogenesis. Su et al. reported that Erianin exerted it anti-angiogenic ability by suppressing HUVEC tube formation through downregulating IDO1 expression (31). Wei et al. also showed that IDO1 expression in breast cancer was associated with tumor microvessel density and poor overall survival of patients (32). In addition, an IDO1 knockout mouse lung metastasis model showed that pulmonary vessel density was significantly reduced (33). These studies demonstrated the role of IDO1 in angiogenesis and our current study is the first to report that miR-153 inhibits IDO1 expression to suppress tryptophan metabolism and angiogenesis. Our current study also revealed that both miR-153 expression and IDO1 knockdown reduced CD34 expression and inhibited HUVEC angiogenesis. This conclusion was also verified in the CAM assay.

Our current study further demonstrated that miR-153's anti-tumor activity in bladder cancer cells was mediated by targeting IDO1, which in turn inactivated the IL6/STAT3/VEGF signaling pathway. Several previous studies showed that STAT3 expression enhanced tumor angiogenesis (34–36). STAT3 can be activated by various cytokines (such as IL6 and non-IL6 family members), which bind to the cell surface receptors resulting in phosphorylation of STAT3 downstream molecules, such as VEGF (37). Indeed, our current data showed that high IDO1 expression promoted IL-6 secretion in bladder cancer cells. Vacher et al. reported that IDO1 induced secretion of IL1B, IL6, IL8, and CXCR4 in breast cancer (38). IL6 expression also promoted tryptophan metabolism to constitute a positive feedback loop (39). In the current study, we found that miR-153 expression reduced IL6 secretion in bladder cancer cells, thereby inhibiting STAT3 signaling and VEGF expression. However, addition of exogenous IL6 to tumor cell culture can restore STAT3 signal transduction and increase VEGF expression. This finding demonstrates that miR-153's anti-angiogenesis activity is regulated through the IL6-mediated STAT3 signaling and targeting of IDO1.

To our knowledge, we are the first to demonstrate that miRNAs can regulate metabolomics in bladder cancer. Specifically, we found that miR-153 expression significantly affected tryptophan metabolism by targeting IDO1 expression and showed a significant association between tryptophan metabolism and tumor growth. In a previous study, Kesarwani et al. showed that the level of tryptophan detected by mass spectrometry was significantly correlated with the efficacy of glioblastoma radiotherapy, and a decrease in tryptophan levels inhibited the reactivation of immune checkpoints (40). Therefore, our future study will determine if there is an association of tryptophan metabolism and miR-153 expression in patient bladder cancer samples.

CONCLUSIONS

In conclusion, our current study demonstrated that miR-153 is significantly downregulated in bladder cancer tissues and cell lines. Overexpression of miR-153 inhibited bladder cell proliferation, migration, invasion, *in vitro* angiogenesis, and *in vivo* tumor cell xenograft formation and growth. Furthermore, we found that IDO1 is a direct target of miR-153, which mediated miR-153 anti-tumor activity in bladder cancer via inactivating the IL6/STAT3/VEGF pathway. These data suggest that miR-153 may be a novel therapeutic target for bladder cancer and further study could provide more novel insights into the mechanisms of bladder cancer progression and metastasis.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

WZ wrote the manuscript and DS generated the figures. ZZ, YG, JZ, YW, LW, RW, and SM contributed to the acquisition of clinical and experimental data. YH and XY contributed to editing the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00619/full#supplementary-material

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Long Non-coding RNA DLEU1 Promotes Cell Proliferation, Invasion, and Confers Cisplatin Resistance in Bladder Cancer by Regulating the miR-99b/HS3ST3B1 Axis

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Li Y, Shi B, Dong F, Zhu X, Liu B and Liu Y (2019) Long Non-coding RNA DLEU1 Promotes Cell Proliferation, Invasion, and Confers Cisplatin Resistance in Bladder Cancer by Regulating the miR-99b/HS3ST3B1 Axis. Front. Genet. 10:280. doi: 10.3389/fgene.2019.00280

Although accumulating evidence has shown the important function of long non-coding RNAs (lncRNAs) in tumor progression and chemotherapy resistance, the role of lncRNA DLEU1 in regulating proliferation, invasion, and chemoresistance of bladder cancer (BCA) cells remains largely unknown. Here, we found that DLEU1 was upregulated in BLCA tissues and BCA patients with high DLEU1 expression exhibited a shorter survival time. Furthermore, mechanistic analysis and functional assays validated that DLEU1 induced cell proliferation, invasion, and cisplatin resistance of BCA cells by de-repressing the expression of HS3ST3B1 through sponging miR-99b. Low miR-99b and high HS3ST3B1 levels were correlated with worse prognosis in patients with BCA. Ectopic expression of HS3ST3B1 or inhibition of miR-99b reversed DLEU1 knockdown-mediated suppression of cell proliferation, invasion, and cisplatin resistance. Thus, our study revealed a novel role for the DLEU1/miR-99b/HS3ST3B1 axis in regulating proliferation, invasion, and cisplatin resistance of BCA cells.

Keywords: long non-coding RNA, DLEU1, bladder cancer, miR-99b, HS3ST3B1

INTRODUCTION

Bladder cancer (BCA) is one of the most common urological malignancies, with an estimated 76,960 new cases and 16,390 deaths in the United States in 2016 (Siegel et al., 2016). Despite advances in the diagnosis and multimodal treatments (including surgery, chemotherapy and radiotherapy), the 5-year survival rate for patients with advanced BCA remains ∼50% (Mohammed et al., 2016). Although cisplatin is commonly used to treat BCA (Dasari and Tchounwou, 2014), the emergence of drug resistance can significantly limit their long-term effectiveness (Chen et al., 2019). The molecular mechanisms underlying BCA progression and chemoresistance are not well understood.

An increasing number of studies have reported that the dysregulation of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) can regulate the progression and chemoresistance of human tumors (Anastasiadou et al., 2018). miRNAs are small, endogenous non-coding coding RNAs that post-transcriptionally modulate gene expression by binding to complementary sequences on particular messenger RNA transcripts (Anastasiadou et al., 2018). lncRNAs are RNA molecules

of greater than 200 nucleotides and can act as molecular sponges, scaffolds, and guides to interact with proteins, mRNAs, or miRNAs, thereby regulating the expression of target genes (Anastasiadou et al., 2018; Dong et al., 2018). Growing evidence indicated that the interactions between lncRNAs and miRNAs play crucial roles in modulating tumor progression and chemoresistance (Cao et al., 2017; Chen et al., 2019).

Long non-coding RNA DLEU1 is overexpressed and promotes migration and invasion in ovarian cancer (Wang et al., 2017), colorectal cancer (Liu et al., 2018), pancreatic cancer (Gao et al., 2018), and lung cancer (Zhang et al., 2019). In this study, we investigated the role of DLEU1 in regulating proliferation, invasion and cisplatin resistance in BCA cells. We found that the expression of DLEU1 was upregulated in BCA tissues compared with normal tissues. The overexpression of DLEU1 predicted poor prognosis of patients with BCA. DLEU1 promoted the proliferation and invasion and conferred resistance to cisplatin by upregulating the expression of oncogene HS3ST3B1 via suppressing miR-99b, a direct inhibitor of HS3ST3B1 in BCA cells. Our findings have demonstrated for the first time that DLEU1 possesses a crucial role in proliferation, invasion, and cisplatin resistance in BCA cells through regulation of the miR-99b/HS3ST3B1 pathway.

MATERIALS AND METHODS

Tissue Samples

Twenty pairs of BCA and adjacent normal tissues were collected from patients with primary BCA at the Fourth Affiliated Hospital of China Medical University, China. None of these patients received chemotherapy or radiotherapy. Samples were immediately frozen in liquid nitrogen and stored at −80°C until used for RNA extraction. Our study was approved by the Research Ethics Committee of Fourth Affiliated Hospital of China Medical University and written informed consents were obtained under the agreement of all patients with pathological confirmation.

Cell Lines, Culture Conditions, and Cell Transfection

Human BCA cell lines (T24 and SW780) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Primary normal human bladder epithelial cells (normal cells) were obtained from The American Type Culture Collection. These cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, United States) supplemented with 5% fetal bovine serum (FBS, Gibco, Grand Island, NY, United States) at 37°C with 5% of CO_2 in a humidified atmosphere. Two different siRNAs against DLEU1 (siRNA-1/2), control siRNA, miR-99b mimic, miR-99b inhibitor and their respective controls were obtained from RiboBio (Guangzhou, China). The DLEU1 expression vector and the HS3ST3B1 expression vector were constructed by Genepharma (Shanghai, China). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, CA, United States) following the manufacturer's protocol.

RNA Extraction and Real-Time PCR Analysis (qRT-PCR)

Total RNA was isolated from BCA samples and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Sample RNA (1 μg) was reverse transcribed using a Reverse Transcription Kit (Takara, Dalian, China). Quantitative real-time PCR was performed using an SYBR Green Real-time PCR Master Mix kit (Toyobo, Osaka, Japan) on the 7900HT fast real-time PCR system (Applied Biosystems, San Francisco, CA, United States). The levels of miR-99b were quantified with the mirVana^{TM} qRT-PCR microRNA Detection Kit (Ambion, Austin, TX, United States). The primers used in this study were synthesized by Genepharma (Shanghai, China). The primer sequences for qRT-PCR were purchase from Genepharma (Shanghai, China). The results were normalized to the levels of *GAPDH* or U6, respectively.

Western Blotting Analysis

Cells were lysed with ice-cold RIPA lysis buffer (Solarbio, Beijing, China) containing Protease Inhibitor Cocktail (Roche, Shanghai, China). Equal amount of protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred on polyvinylidene difluoride membrane (Millipore, Bedford, MA, United States) and probed by antibodies against HS3ST3B1 (Sigma-Aldrich Co., St. Louis, MO, United States, 1:1000) and GAPDH (Santa Cruz, CA, United States, 1:5000). Following incubation with the corresponding secondary antibodies, signals were detected with the ECL detection kit (Pierce, Rockford, IL, United States).

CCK-8 Assay

Five thousand cells were seeded into 96-well plates and transfected with DLEU1 siRNA, control sRNA, DLEU1 expression vector or control vector for 72 h. Cell viability was measured using the CCK-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Colony Formation Assay

Colony formation assay was conducted as reported (Ihira et al., 2017).

Apoptosis Assay

Three thousand cells were seeded in 96-well plates and transfected as indicated. After 24 h of incubation, cells were treated with saline or varying doses of cisplatin (Sigma-Aldrich Co., St. Louis, MO, United States). After 24 h of treatment, cell viability was determined using the CCK-8 (Dojindo, Kumamoto, Japan). Values obtained were expressed as the percentage of surviving cells, with the viability of saline-treated cells set at 100%. Cell apoptosis was measured by the Caspase-Glo 3/7 assay reagent (Promega, Madison, WI, United States) as described (Dong et al., 2016).

Cell Invasion Assay

Transwell cell invasion assays were performed using Boyden chambers (Corning, New York, NY, United States) that use 8 μm pore membranes with Matrigel as reported (Xiong et al., 2017).

In brief, 1×10^5 cells were added to a Matrigel invasion chamber and FBS was added to the lower chamber. After 24 h, the non-invading cells were gently removed with a cotton swab. Invaded cells were stained with 1% toluidine blue solution and counted.

Luciferase Reporter Assay

The fragment of DLEU1 or HS3ST3B1 3′-UTR (wild-type: WT; mutant: MUT) containing the miR-99b binding site was synthesized and then cloned into the pGL3-basic vector (Promega, Madison, WI, United States). Cells were seeded into 24-well plates. Each luciferase reporter vector was co-transfected with pRL-CMV (Promega, Madison, WI, United States) expressing Renilla luciferase, and miR-99b mimic, miR-99b inhibitor or their respective controls using Lipofectamine 2000 reagent (Invitrogen, CA, United States). After 48 h, cell lysates were made. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States) according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of Renilla luciferase activity for each sample.

RNA Immunoprecipitation Assay (RIP)

The RIP assay was performed with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, United States) following the manufacturer's protocol. Briefly, cells were collected and lysed using RIP lysis buffer. One hundred microliters of cell extract was incubated with RIP buffer containing magnetic beads conjugated to an anti-Argonaute2 (Ago2) antibody (Millipore, Bedford, MA, United States) or negative control IgG (Millipore, Bedford, MA, United States). The samples were incubated with Proteinase K to digest proteins and then the immunoprecipitated RNA was isolated. The purified RNA was subjected to quantitative PCR to detect the presence of DLEU1 or miR-99b. The total RNAs were the input controls.

Statistical Analysis

The results are the means ± standard deviation from at least three experiments. Statistical evaluations were carried out with SPSS 22.0 software (Chicago, IL, United States). Differences between two groups were compared using Student's *t*-test, and differences among three groups were analyzed by ANOVA. *P*-values <0.05 were considered significant.

RESULTS

lncRNA-DLEU1 Is Elevated in BCA Tissues and High DLEU1 Expression Predicts Poor Prognosis of BCA Patients

In order to investigate the relevance of DLEU1 in BCA development, we first checked its expression using the TCGA BCA dataset through the UALCAN web portal (Chandrashekar et al., 2017). As shown in **Figure 1A**, the expression of DLEU1 in BCA tissues was significantly higher than that in normal tissues. Then, the expression levels of DLEU1 were examined in 20 pairs of BCA tissues and adjacent normal tissues. Our

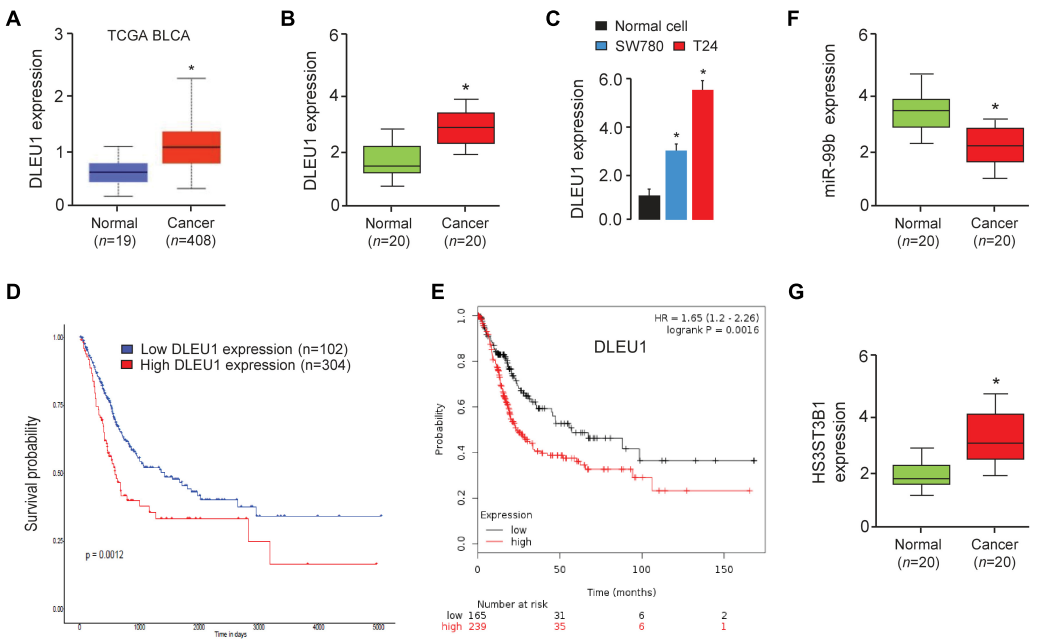

FIGURE 1 | DLEU1 is upregulated in BCA tissues and high DLEU1 expression predicts poor disease prognosis. **(A)** The expression of DLEU1 in normal and BCA tissues from the TCGA database UALCAN. **(B)** qRT-PCR analysis of DLEU1 expression in BCA samples ($n = 20$) and adjacent normal tissues ($n = 20$). **(C)** qRT-PCR analysis of DLEU1 expression in BCA cell lines and normal cells. **(D,E)** High expression of DLEU1 decreases survival probability. The publicly available TCGA datasets were obtained from the web portal of UALCAN **(D)** and KMplotter **(E)**. **(F)** qRT-PCR analysis of miR-99b expression in BCA samples ($n = 20$) and adjacent normal tissues ($n = 20$). **(G)** qRT-PCR analysis of HS3ST3B1 expression in BCA samples ($n = 20$) and adjacent normal tissues ($n = 20$). $^*P < 0.05$.

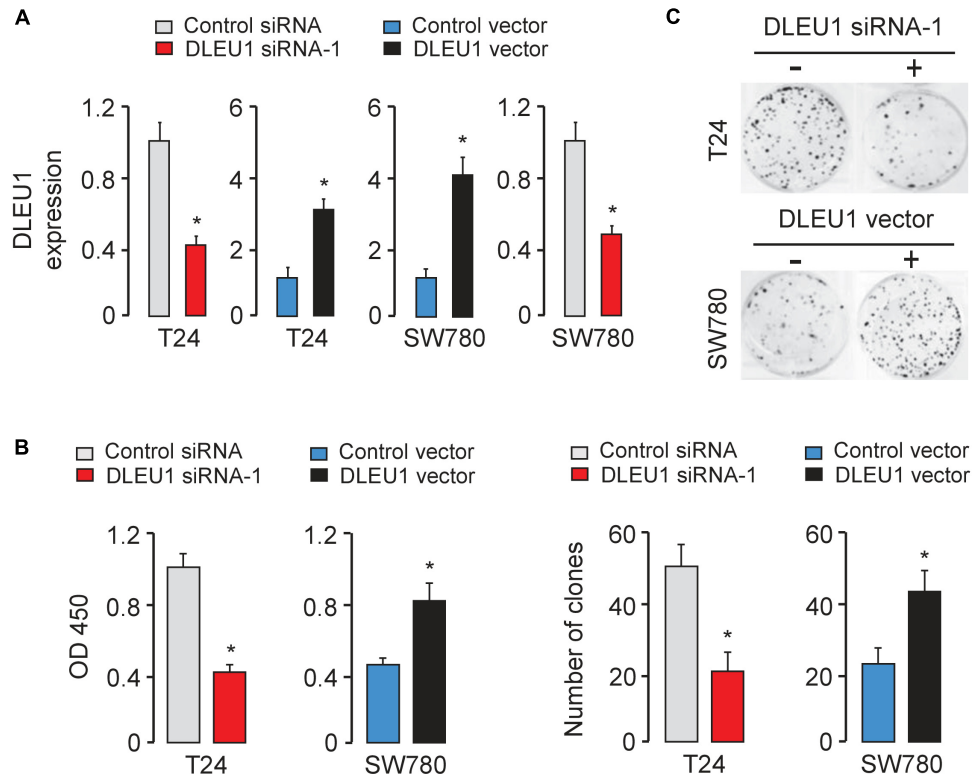

FIGURE 2 | Knockdown of DLEU1 inhibits BCA cell proliferation and invasion *in vitro*. **(A)** Validation of the transfection efficiency in BCA cell lines using qRT-PCR analysis. **(B)** DLEU1 induces cell proliferation as measured by CCK-8 assays in T24 and SW780 cells. **(C)** DLEU1 promotes colony formation as demonstrated by colony formation assays in T24 and SW780 cells. $^*P < 0.05$.

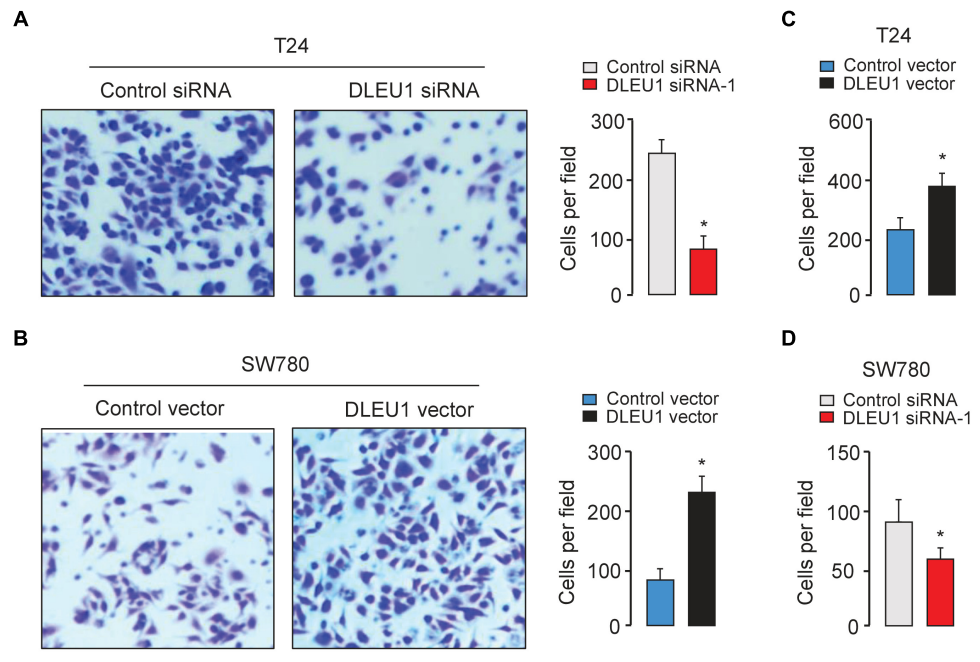

FIGURE 3 | DLEU1 promotes cell invasion in BCA cells. Representative images (left) and bar graphs (right) depicting the invasion ability of T24 **(A)** and SW780 **(B)** cells after the knockdown or overexpression of DLEU1, and of T24 cells **(C)** and SW780 cells after the overexpression or knockdown of DLEU1 **(D)**. $^*P < 0.05$.

qRT-PCR analysis demonstrated that DLEU1 was upregulated in BCA samples compared to adjacent normal tissues (**Figure 1B**). The expression of DLEU1 was measured in two BCA cell lines (SW780 and T24) and normal cells. Higher levels of DLEU1 were detected in SW780 and T24 cells as compared with normal cells (**Figure 1C**). Through the web tools, including UALCAN and the KMplotter (Győrffy et al., 2013), we found that higher levels of DLEU1 were positively correlated with reduced patient survival (**Figures 1D,E**). These findings demonstrated that DLEUU1 may play an important role in the regulation of BCA development and may serve as a prognostic marker in BCA.

DLEU1 Acts as an Oncogene by Promoting the Proliferation and Invasion of BCA Cells

Next, we explored the function of DLEU1 in tumorigenesis and development of BCA. Because T24 cells have relatively high expressions of DLEU1, whereas SW780 cells have relatively low expressions of DLEU1 (**Figure 1B**), we silenced the expression of endogenous DLEU1 in T24 cells using two different siRNAs, and overexpressed DLEU1 in SW780 cells by using an expression vector containing human DLEU1. In addition, we also overexpressed DLEU1 in T24 cells and silenced the expression of DLEU1 in SW780 cells. The qRT-PCR analysis was used to confirm the transfection efficiency (**Figure 2A** and **Supplementary Figure 1A**).

The CCK-8 and colony formation assay demonstrated that the knockdown of DLEU1 attenuated the proliferation of T24 cells, and overexpression of DLEU1 significantly enhanced the proliferation of SW780 cells (**Figures 2B,C** and **Supplementary Figure 1B**). We examined cell invasion by transwell assay after the knockdown or overexpression of DLEU1. We observed that the invasive abilities of T24 cells were remarkably reduced upon silencing of DLEU1 compared with the control cells (**Figure 3A** and **Supplementary Figure 1C**). Meanwhile, the invasion of SW780 cells was significantly induced following upregulation of DLEU1 expression as shown by transwell assay (**Figure 3B**). Moreover, the upregulation of DLEU1 in T24 cells enhanced cell invasion, and downregulation of DLEU1 in SW780 cells decreased cell invasion (**Figures 3C,D**). Taken together, these findings indicated the growth and invasion-stimulating roles of DLEU1 in BCA.

DLEU1 Confers Resistance to Cisplatin-Induced Apoptosis

Next, we evaluated the effect of DLEU1 on cisplatin sensitivity in BCA cells. Following the knockdown or overexpression of DLEU1, BCA cells were treated with cisplatin and then assayed for their sensitivity to cisplatin using the CCK-8 assay. Our results showed that DLEU1 silencing increased the sensitivity to cisplatin (**Figure 4A**) and enhanced the cisplatin-induced cell apoptosis, as determined by an increase in the activities of caspase-3/7 in T24 cells (**Figure 4B**). Conversely, ectopic overexpression of DLEU1 endowed SW780 cells with refractoriness to cisplatin (**Figure 4A**) and antagonized cisplatin-induced apoptosis (**Figure 4B**). We next explored whether the modulation of DLEU1 alter the expression of caspase-3 in BCA cells using qRT-PCR analysis. Our results showed that the levels of caspase-3 was significantly

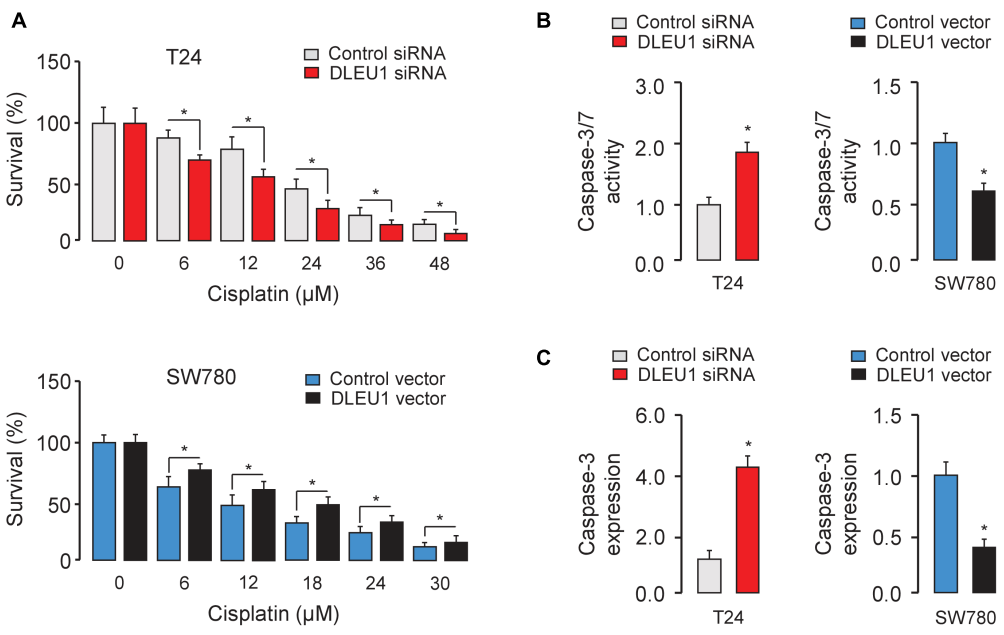

FIGURE 4 | Knockdown of DLEU1 enhances the sensitivity of BCA cells to cisplatin-induced apoptosis. **(A)** T24 (upper panel) and SW780 (bottom panel) cells were transfected as indicated for 24 h, and then cells were incubated with or without cisplatin for another 24 h. Cell viability was detected using CCK-8 assays. **(B)** The effects of cisplatin on apoptosis of BCA cells were measured using Caspase-Glo 3/7 assays. **(C)** qRT-PCR analysis of caspase-3 expression in T24 and SW780 cells after the knockdown or overexpression of DLEU1. $^*P < 0.05$.

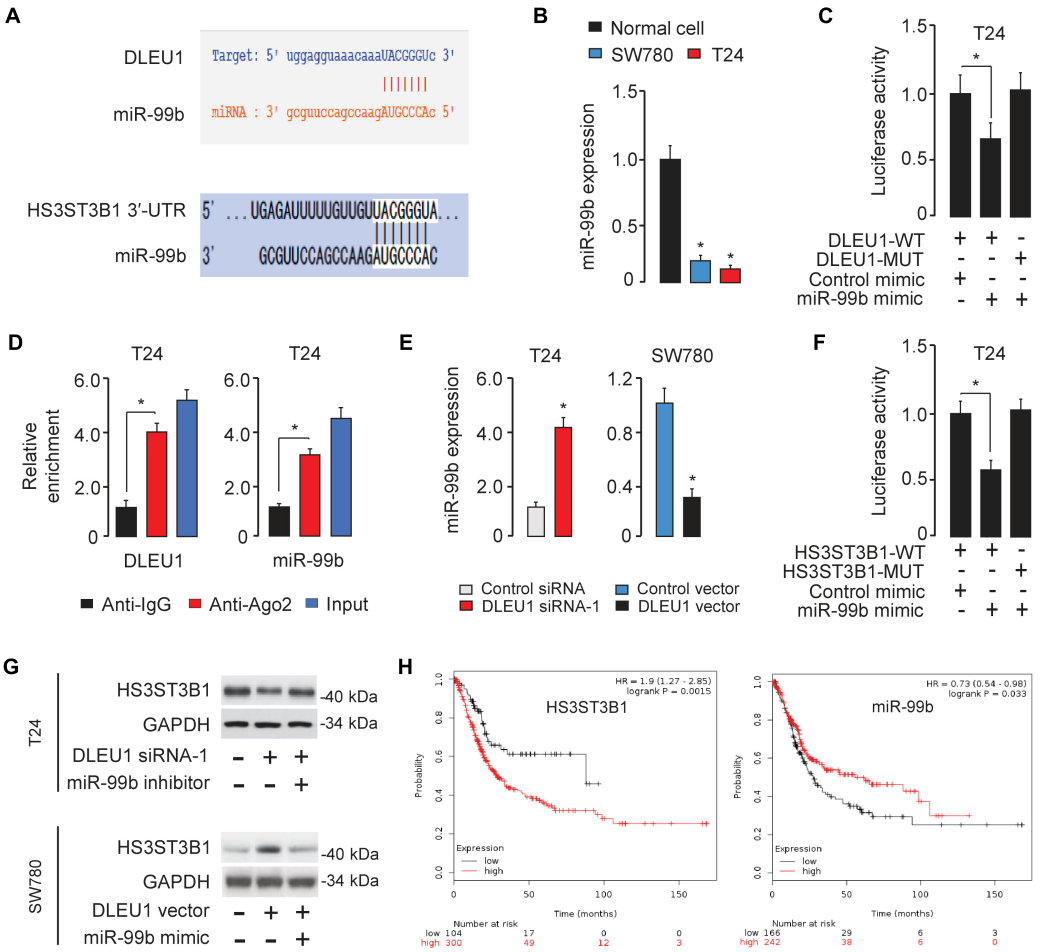

FIGURE 5 | DLEU1 sponges miR-99b to upregulate HS3ST3B1 expression. **(A)** The miR-99b-binding sequences in DLEU1 and HS3ST3B1 3′-UTR. **(B)** qRT-PCR analysis of miR-99b expression in BCA cell lines and normal cells. **(C)** Luciferase reporter assay in T24 cells, co-transfected with a luciferase reporter plasmid containing wild-type (WT) or mutant (MUT) DLEU1 and the indicated miRNAs. **(D)** RIP assay showed the enrichment of DLEU1 and miR-499b in Ago2-containing beads. **(E)** qRT-PCR analysis of miR-99b expression in BCA cells after the knockdown or overexpression of DLEU1. **(F)** Luciferase reporter assay in T24 cells, co-transfected with a luciferase reporter plasmid containing wild-type (WT) or mutant (MUT) HS3ST3B1 3′-UTR and the indicated miRNAs. **(G)** The protein expression of HS3ST3B1 in T24 cells co-transfected with DLEU1 siRNA or control siRNA, together with miR-99b inhibitor or its respective control were analyzed using western blotting analysis (upper panel). The protein expression of HS3ST3B1 in SW780 cells co-transfected with DLEU1 expression vector or control vector, together with miR-99b mimic or its respective control were examined using western blotting analysis (bottom panel). **(H)** Kaplan–Meier survival analysis of overall survival in BCA patients based on HS3ST3B1 or miR-99b expression. The publicly available TCGA datasets were obtained from the web portal of KMplotter. $^*P < 0.05$.

increased after the knockdown of DLEU1 in T24 cells, while was significantly repressed after the overexpression of DLEU1 in SW780 cells (**Figure 4C**). Consequently, these results indicated the possibility that the knockdown of DLEU1 sensitized BCA cells to cisplatin-induced apoptosis.

DLEU1 Sponges miR-99b and Indirectly Upregulates HS3ST3B1 Expression

Long non-coding RNAs can function as competing endogenous RNAs to modulate the expression and biological functions of miRNAs. Thus, we speculated that DLEU1 might promote BCA tumorigenesis, progression and cisplatin resistance by suppressing the functions of certain miRNAs. The potential miRNA targets of DLEU1 were predicted by the bioinformatics database StarBase V2.0 (Li et al., 2014). We identified the miR-99b-binding site in DLEU1 (**Figure 5A**). Interestingly, the expression level of miR-99b was significantly reduced in BCA samples compared to normal samples (**Figure 1F**), and BCA cell lines had lower levels of miR-99b than the normal cells (**Figure 5B**). These data suggested that the expression of miR-99b was negatively correlated with that of DLEU1 in BCA cells.

To further explore the relationship between DLEU1 and miR-99b, a dual-luciferase reporter assay was performed. The transfection with miR-99b mimic significantly reduced the luciferase activity of wild-type DLEU1 reporter vector, but not that of mutant reporter vector in T24 cells (**Figure 5C**), suggesting that DLEU1 was directly targeted by miR-99b in BCA

cells. In order to further verify whether DLEU1 and miR-99b are in the same RNA-induced silencing complex, anti-Ago2 RIP assay was performed in T24 cells. The expression level of DLEU1 and miR-99b was higher in the anti-Ago2 group than that in the anti-normal IgG group (**Figure 5D**). Importantly, the knockdown of DLEU1 elevated the levels of miR-99b, and the overexpression of DLEU1 reduced the expression levels of miR-99b in T24 cells (**Figure 5E**), indicating that DLEU1 and miR-99b mutually regulate each other. Taken together, the results suggested that DLEU1 interacts with miR-99b and regulates its expression.

We further searched for the target genes of miR-99b using a bioinformatics program TargetScan, and found that *HS3ST3B1* mRNA contained a potential target site of miR-99b in its 3′-UTR (**Figure 5A**). Previous studies have shown that HS3ST3B1 was upregulated in several tumors and can maintain the mesenchymal phenotype and promote cancer cell proliferation and angiogenesis (Song et al., 2011; Zhang et al., 2015, 2018; Hellec et al., 2018). Our qRT-PCR analysis showed that HS3ST3B1 expression was significantly upregulated in BCA samples compared to adjacent normal samples (**Figure 1G**). Dual-luciferase reporter assays demonstrated that the transfection with miR-99b mimic attenuated the luciferase activities of the wild-type HS3ST3B1 3′-UTR, but had no effect on the mutant 3′-UTR of HS3ST3B1 (**Figure 5F**), verifying the direct binding between miR-99b and HS3ST3B1 in BCA cells.

Furthermore, the protein levels of HS3ST3B1 in T24 cells were reduced after transfecting with DLEU1 siRNA-1 (**Figure 5G**). This inhibitory effect of DLEU1 silencing was notably reversed by co-transfection with miR-99b inhibitor in T24 cells (**Figure 5G**). In SW780 cells, the expression of HS3ST3B1 was significantly increased after transfecting with the DLEU1 expression vector (**Figure 5G**). This increase in HS3ST3B1 expression was reversed by co-transfection with miR-99b mimic (**Figure 5G**). The relationship between HS3ST3B1 or miR-99b expression and patient survival was analyzed in a TCGA BCA cohort using the KMplotter web tool. We found that higher HS3ST3B1 levels or lower miR-99b levels were significantly correlated with decreased overall survival (**Figure 5H**). These results suggested that DLEU1 functions as a competing endogenous RNA by sponging miR-99b and indirectly upregulated HS3ST3B1 expression in BCA cells.

DLEU1 Promotes Proliferation, Invasion, and Chemoresistance by Upregulating HS3ST3B1 Expression Through Protecting It From miR-99b-Mediated Suppression

To test whether DLEU1 exerts oncogenic functions in BCA by modulating the miR-99b/HS3ST3B1 axis, we investigated the effects of miR-99b or HS3ST3B1 on DLEU1-induced cancer cell proliferation, invasion, and cisplatin resistance. We observed that the knockdown of the endogenous DLEU1 significantly weakened the proliferation, invasion and enhanced the sensitivity of T24 cells to cisplatin treatment (**Figure 6**). These influences of DLEU1 knockdown were reversed by down-regulation of miR-99b or ectopic expression of HS3ST3B1 in T24 cells (**Figure 6**). Collectively, these data supported the hypothesis that DLEU1 promotes BCA cell proliferation, invasion, and cisplatin resistance, at least partly, through sponging miR-99b and then upregulating HS3ST3B1 expression.

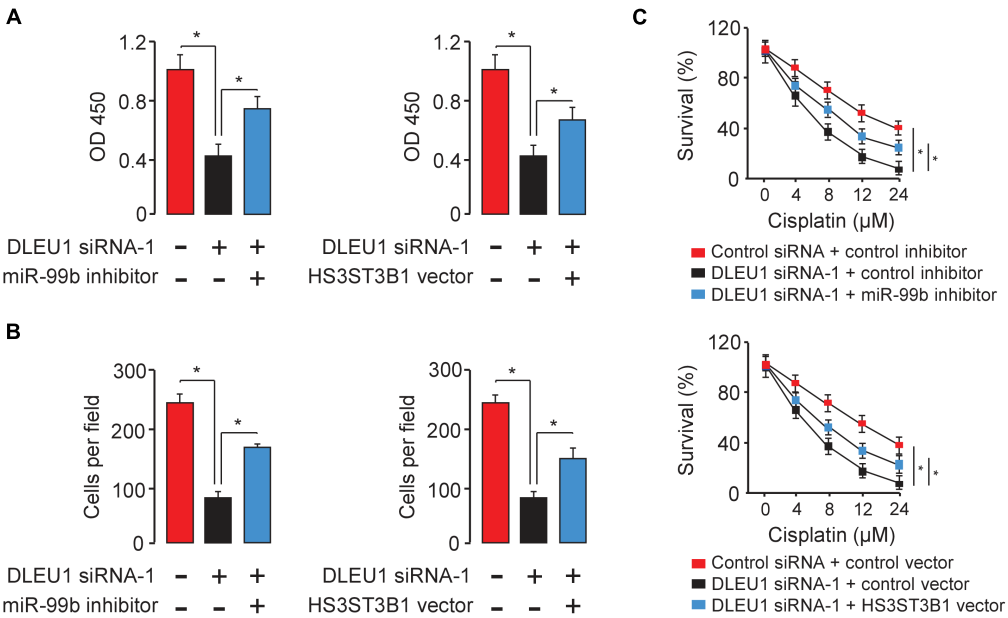

FIGURE 6 | DLEU1 promotes BCA cell proliferation, invasion, and cisplatin resistance by sponging miR-99b and restoring HS3ST3B1 expression. Decreased cell proliferation **(A)**, invasion **(B)**, and cisplatin resistance **(C)** upon DLEU1 knockdown in T24 cells were rescued by miR-99b silencing or ectopic expression of HS3ST3B1. $*P < 0.05$.

DISCUSSION

Long non-coding RNAs have important roles in various types of malignant tumors, and some lncRNAs may function as novel diagnostic markers and therapeutic targets in BCA (Quan et al., 2018; Taheri et al., 2018; Wieczorek and Reszka, 2018). DLEU1 is aberrantly overexpressed in a variety of tumors and contributes to tumorigenesis and cancer development (Wang et al., 2017; Gao et al., 2018; Liu et al., 2018; Zhang et al., 2019). In this study, we demonstrated for the first time that, the expression of DLEU1 is significantly increased in BCA tissues and BCA cell lines, and elevated expression of DLEU1 predicts worse patient survival. Subsequent functional investigation revealed that DLEU1 promotes BCA cell growth, invasion and induces cisplatin resistance. Furthermore, we showed that DLEU1 executes its tumor-promoting functions by sponging tumor suppressor miR-99b and increasing the protein expression of oncogene HS3ST3B1 in BCA.

To investigate the underlying mechanisms of DLEU1, we have identified miR-99b as a direct target of DLEU1 in BCA cells. Dual-luciferase reporter and RIP assay demonstrated the direct interaction between DLEU1 and miR-99b. Emerging studies have shown that miR-99b was downregulated and served as a novel tumor suppressor in many cancers, including lung cancer (Kang et al., 2012), cervical cancer (Li et al., 2018), colorectal cancer (Li et al., 2015), and gastric cancer (Wang et al., 2018). However, miR-99b was shown to induce cancer cell migration and invasion and metastasis in esophageal squamous cell carcinoma (Ma et al., 2017). Currently, the function of miR-99b in BCA remains unclear. We showed here that the downregulation of miR-99b was associated with poor survival in patients with BCA, and miR-99b can partly reverse DLEU1-induced cell proliferation, invasion, and cisplatin resistance, suggesting that miR-99b has important roles in suppressing BCA progression and overcoming cisplatin resistance in BCA cells.

MicroRNAs could regulate the expression of multiple target genes simultaneously. In the present study, we found that miR-99b bound directly to HS3ST3B1 in BCA cells. HS3ST3B1 was overexpressed in tumors and participated in regulating the invasive phenotype, cell proliferation, and angiogenesis (Song et al., 2011; Zhang et al., 2015, 2018; Hellec et al., 2018), but little was known about its function in BCA. Here, we showed that high HS3ST3B1 levels were correlated with a shorter survival time of BCA patients, and the ectopic expression of HS3ST3B1 could promote the proliferation and invasion of BCA cells, and enhance the resistance of BCA cells to cisplatin treatment. Taken together, these data suggested that HS3ST3B1 has diverse oncogenic roles in promoting the proliferation, invasion and chemoresistance in BCA cells.

CONCLUSION

In conclusion, the present study showed that DLEU1 is upregulated in BCA and promotes BCA cell proliferation, invasion, and cisplatin resistance via competitively sponging miR-99b and then restoring HS3ST3B1 expression. Therefore, the DLEU1/miR-99b/HS3ST3B1 axis represents a key pathway involved in BCA progression and cisplatin resistance, and DLEU1 could be considered as a potential target for BCA therapies in the future.

DATA AVAILABILITY

All data generated during this study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

YiL designed the experiments. YoL and BS performed the experiments. FD, XZ, and BL made significant revisions to the manuscript. All authors read and approved the final manuscript.

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.2019.00280/full#supplementary-material

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MiR-486-5p Serves as a Good Biomarker in Nonsmall Cell Lung Cancer and Suppresses Cell Growth With the Involvement of a Target PIK3R1

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MicroRNAs are a class of noncoding RNAs that can be involved in the regulation of gene expression in cancers, including lung cancer. Our previous research has shown that miR-486-5p is one of the most downregulated microRNAs in tissue and serum samples of lung cancer as a good diagnostic biomarker. The objective of this study is to investigate the roles of miR-486-5p in the progression of lung cancer. In this study, miR-486-5p was further validated to be significantly downregulated in additional nonsmall cell lung cancer (NSCLC) tissue, serum, and cell samples by quantitative reverse transcription-polymerase chain reaction (RT-PCR), and the expression level of miR-486-5p was significantly associated with clinical phenotype of NSCLC. The PIK3R1 gene was confirmed to be a direct target of miR-486-5p by dual-luciferase reporter assay, and the expression level of miR-486-5p was inversely correlated with that of PIK3R1 in tumor tissues ($r = -0.774$, $p < 0.01$). Overexpressed miR-486-5p effectively inhibited cell proliferation and invasion and successfully induced apoptosis *in vitro*. PIK3R1 was involved in the suppression of miR-486-5p on cell growth. It can be concluded that miR-486-5p may act as a tumor suppressor contributing to the progression of NSCLC, and miR-486-5p would be a diagnostic and prognostic biomarker and a potential therapeutic target for lung cancer.

Keywords: nonsmall cell lung cancer, miR-486-5p, PIK3R1, biomarker, proliferation

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INTRODUCTION

Lung cancer is the most common incident cancer and the leading cause of cancer-related death, with 733,300 new cases and 610,200 deaths in China in 2015 (Chen et al., 2016). Lung cancer patients were frequently diagnosed at advanced stages, resulting in a high mortality. Nonsmall cell lung cancer (NSCLC) is the most common subtype of lung cancer, including two main histologic subtypes: lung squamous cell carcinoma (SCC) and lung adenocarcinoma (AC) (Travis et al.,

Abbreviations: miRNA, MicroRNA; NSCLC, Nonsmall cell lung cancer; SCC, squamous cell carcinoma; AC, adenocarcinoma; UTR, untranslated region; CCK-8, Cell Counting Kit-8; NC, negative control; V-FITC, annexin V fluorescein isothiocyanate; PI, propidium iodide; siRNAs, small interfering RNAs.

2015). In recent years, the early diagnosis and targeted therapy of lung cancer attracted more attention, and studies shown that molecular biomarkers have been discovered in different tumors and could be well applied in the early diagnosis, prognosis, and therapy of lung cancer (Zhang et al., 2016a; Best et al., 2017).

MicroRNAs (miRNAs) are endogenous noncoding small RNAs (~22 nt) that modulate the activity of mRNA by hybridizing to complementary sequences in the 3'-untranslated region (UTR) of specific targets (Bartel, 2004; Kloosterman and Plasterk, 2006). A lot of studies have demonstrated that miRNAs could participate in various cell biological processes, including cell growth, differentiation, and cell apoptosis (Ambros, 2003; Kloosterman and Plasterk, 2006; Stefani and Slack, 2008). Moreover, studies have shown that miRNAs are frequently dysregulated in cancers, including NSCLC (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Kumar et al., 2008). Recently, miR-486-5p has been found to function as a potential suppressive gene in different cancers, including lung cancer (Peng et al., 2013; Wang et al., 2014; Liu et al., 2016; Zhang et al., 2016b). Coincidentally, our previous study found aberrant miR-486-5p expression in both tissue and serum samples of NSCLC by Hiseq2500 sequencing; miR-486-5p might be an early diagnostic biomarker for NSCLC (Tian et al., 2016). However, the opposite expression levels and roles of miR-486-5p in lung cancer were identified in different reports (Yu et al., 2010; Tan et al., 2011; Jin et al., 2017; Sromek et al., 2017). Therefore, we need to further evaluate and validate the biological function of miR-486-5p in the progression of NSCLC in this study and whether miR-486-5p have an important role in NSCLC by targeting other functional genes?

In the current study, the expression level of miR-486-5p was further confirmed to be significantly lower in NSCLC tissue, serum, and cell lines than in the corresponding controls, and low expression of miR-486-5p was significantly inversely correlated with advanced tumor tumor-mode-metastasis(TNM) stage and larger tumor size in NSCLC tissue. Overexpressed miR-486-5p significantly restrained proliferation and invasion of A549 and H1299 cells and induced cell apoptosis. PIK3R1 was involved in the suppression of miR-486-5p on cell growth. Therefore, miR-486-5p may act as a potent tumor suppressor of cellular growth and migratory capacity in NSCLC.

MATERIALS AND METHODS

Clinical Tissue and Serum Specimens

A total of 36 paired surgical cancerous, normal tissue and serum samples were obtained from NSCLC patients, and the 36 normal serum samples were obtained from healthy volunteers. Another 39 tumor tissue samples were also collected for clinical pathology analysis by qRT-PCR (**Supplementary Table 1**). All samples mentioned were obtained from Jiangsu Province People's Hospital with informed consent. This study was approved by the Ethics Committee of Jiangsu Province People's Hospital. All tissue and serum specimens were stored at −80°C for subsequent RNA extraction.

Cell Culture

The human NSCLC cell line A549, H1299, and normal pulmonary epithelial cell line BEAS-2B and human renal epithelial cell line 293T were obtained from the Cell Bank, China Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Corning, USA) supplemented with 10% (v/v) fetal bovine serum (Biological Industries, Israel) at 37°C in 5% CO_2 atmosphere.

RNA Extraction and Quantitative RT-PCR

Tissue and cell RNAs were isolated using mirVana™ miRNA isolation Kit (Ambion, USA); serum RNA was isolated according to the protocol of miRNeasy Serum/Plasma Kit (Qiagen, Germany). MiRNA and mRNA analysis were performed by qRT-PCR using PrimeScript™ II reverse transcriptase (Takara, Japan) and SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer's protocols. Relative quantification of miR-486-5p and PIK3R1 were obtained by normalization to U6 snRNA and β-Actin, respectively. The qRT-PCR experiments were repeated at least three times. The expression levels of miR-486-5p and PIK3R1 were calculated by ΔΔCt, normalized with $\Delta Ct = AvgCt_{miR\text{-}486\text{-}5p} - AvgCt_{U6}$ or $AvgCt_{PIK3R1} - AvgCt_{\beta\text{-}Actin}$. The primers used in this study are listed in **Supplementary Table 2**.

Bioinformatics

The target genes of miR-486-5p were predicted by the intersection of five prediction software packages, microT, miRmap, TargetScan, PicTar, and miRanda, based on starBase v3.0 (Yang et al., 2011; Li et al., 2014). The selected PIK3R1 contained regions in the 3'-UTRs that perfectly matched the seed sequence of miR-486-5p. The Cancer Genome Atlas (TCGA) data were employed to analyze the expression level of miR-486-5p in lung SCC and AC. The *PI3K-Akt signaling pathway* and *nonsmall cell lung cancer pathway* were analyzed in the KEGG database (https://www.kegg.jp/). The Kaplan–Meier plotter was adopted for the prognosis analysis of miR-486-5p in NSCLC (Lanczky et al., 2016; Nagy et al., 2018).

PIK3R1 3'-UTR Luciferase Reporter Assay

The human PIK3R1 3'-UTR and luciferase reporter construct were synthesized by cloning the human PIK3R1 3'-UTR sequence into a GP-miRGLO report vector (Sangon Biotech, China). The PIK3R1 3'-UTR wild type, mutant type, and a positive control (miR-486-5p inhibitor sponge) on binding sites were directly chemo-synthesized (**Supplementary Table 3**). The synthesized fragment was cloned into GP-miRGLO luciferase report vector at SacI and XhoI sites. The sequence of plasmid was confirmed by DNA sequencing. The 293T cells (5×10^5 per well) were seeded in a 12-well plate the day before transfection, and then cotransfected with firefly luciferase 3'-UTR (GP-miRGLO-PIK3R1), pRL-TK-Renilla-luciferase plasmid (Promega, USA), and miR-486-5p mimics or negative control (NC). After 24 and 48 h, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, USA), respectively. Firefly luciferase activity was normalized to *Renilla* luciferase activity for transfection efficiency. Experiments were repeated at least three times.

Lentivector Infection, Transfection, and siRNA Silencing

To force the expression of miR-486-5p in NSCLC cells, the pre-miR-486-5p construct (sequence shown in **Supplementary Table 3**) was packaged with a pGLV2-U6 lentivector packaging plasmid mix (Sangon Biotech, China) in a 293T packaged cell line. The Transdux reagent (System Bioscience, USA) was used for virus transduction, and infected cells were selected by ampicillin (Sigma, USA). Transfection of pre-miR-486-5p expression vector was carried out with Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen, USA), and transfected A549 and H1299 cells were selected by penicillin and streptomycin (Gibco, USA). Similarly, the lentivector packaging plasmid was inserted PIK3R1 sequence to overexpress PIK3R1.

Two small interfering RNAs (siRNAs) specifically against PIK3R1 (si-739 and si-1178) were designed, and the sequences are shown in **Supplementary Table 3**. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's protocol with si-PIK3R1 or scrambled sequences. At least three independent experiments were carried out.

Western Blot

Total proteins (50–100 μg) extracted from cell lines were analyzed by SDS–polyacrylamide gel electrophoresis and were transferred electrophoretically to a PVDF membrane (Millipore, USA). To evaluate the expression of PIK3R1 (p85α), blots were blocked with 5% nonfat milk in Tris-buffered saline and Tween 20 and incubated with a primary rabbit anti-PIK3R1 polyclonal antibody, PAB19524 (Abnova). The polyclonal PAB19524 antibody was produced by immunizing animals with a synthetic peptide corresponding to amino acids at N-terminus of human PIK3R1. Antibody for GAPDH, G8795 (Sigma, USA), was used as a control. The blots were then reprobed with secondary antibody and detected with SuperSignal West Pico Chemiluminescent Substrates System (Pierce, USA).

Cell Proliferation, Transwell, and Annexin V Apoptosis Assay

Transfected cells were measured for cell proliferation with Cell Counting Kit-8 (CCK-8, Dojindo, Japan) after incubating for 24, 48, and 72 h in a 96-well microplate (Corning, USA). Each well was added with 5 μl CCK-8 and 100 μl fresh medium, then incubated for 2 h. The absorbance was measured at 450 nm by Multiskan FC (Thermo Fisher, USA). The A549 and H1299 cells without any transfection were analyzed as the blank controls, and the cells transfected with empty vector were the NC.

To determine cell invasion and migration, cells were plated in medium with 1% serum in the top chamber of a Transwell (Corning, USA) after transfection. The bottom chamber contained standard medium with 20% fetal bovine serum. After 72-h incubation, the cells that had migrated to the lower surface of the membrane were fixed with cold ethyl alcohol, stained with 0.1% crystal violet, and photographed under a microscope. Cell numbers were counted under a light microscope at ×400 magnification. Experiments were carried out at least three times.

Cells were stained with annexin V fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) using the Annexin V-FITC Kit (Beckman Coulter, USA) for flow cytometric analysis (FACSCalibur, BD Bioscience, USA). The apoptotic index was calculated as the percentage of annexin V+/PI cells.

Statistical Analysis

The differences of two groups were analyzed using paired *t*-test with one-tailed *p* value. One-way ANOVA was used to determine the difference in miR-486-5p expression in three groups of different clinicopathological factors. Pearson correlation analysis was used to determine the correlation between miR-486-5p and PIK3R1 expression with two-tailed *p* value. In all cases, $p < 0.05$ was considered statistically significant difference.

RESULTS

MiR-486-5p Was Significantly Downregulated and Associated With Clinical Phenotype and Survival Data in NSCLC

A previous study has reported that miR-486-5p was significantly downregulated in NSCLC tissue and serum compared to the normal tissue and serum, and the Hiseq 2500 sequencing and qRT-PCR results showed good consistency (Tian et al., 2016). In this study, we further validated the low expression of miR-486-5p in additional 36 paired tissue ($p < 0.01$, **Figure 1A)** and serum samples ($p < 0.05$, **Figure 1B**) of NSCLC compared with corresponding controls by qRT-PCR. The TCGA data showed miR-486-5p was significantly lower expressed in lung SCC (322 samples, $p < 0.01$) and AC tissue (430 samples, $p < 0.01$) compared to the controls (**Supplementary Figure 1**). These results illustrated that the downregulated miR-486-5p might be associated with NSCLC carcinogenesis as a good diagnostic biomarker.

To further determine the relations of clinicopathological factors (**Supplementary Table 1**) with miR-486-5p expression, we evaluated the levels of miR-486-5p in another 39 tumor tissue samples by qRT-PCR. The results revealed that the expression level of miR-486-5p was statistically decreased in stages III and II compared to stage I ($p < 0.001$, **Figure 1C**). Moreover, the relative expression level of miR-486-5p was significantly lower in tumor with size ≥5 or 30–50 mm than in size ≤30 mm ($p < 0.001$, **Figure 1D**). Low expression of miR-486-5p was inversely correlated with advanced tumor TNM stage and larger tumor size in NSCLC tumor tissues. However, there were no significant correlations in miR-486-5p expression levels and patients' age, gender, smoking status, or histological type (**Supplementary Table 1**). From the Kaplan–Meier plotter, low expression of miR-486-5p had significantly worse prognosis in lung SCC patients (472 samples, $p = 0.044$). However, there was no significance between its expression and prognosis data in lung AC patients (504 samples, $p = 0.08$, **Figure 2**). It could be inferred that the low miR-486-5p expression was closely correlated to the progression and prognosis of NSCLC, and miR-486-5p might be a tumor suppressor in NSCLC.

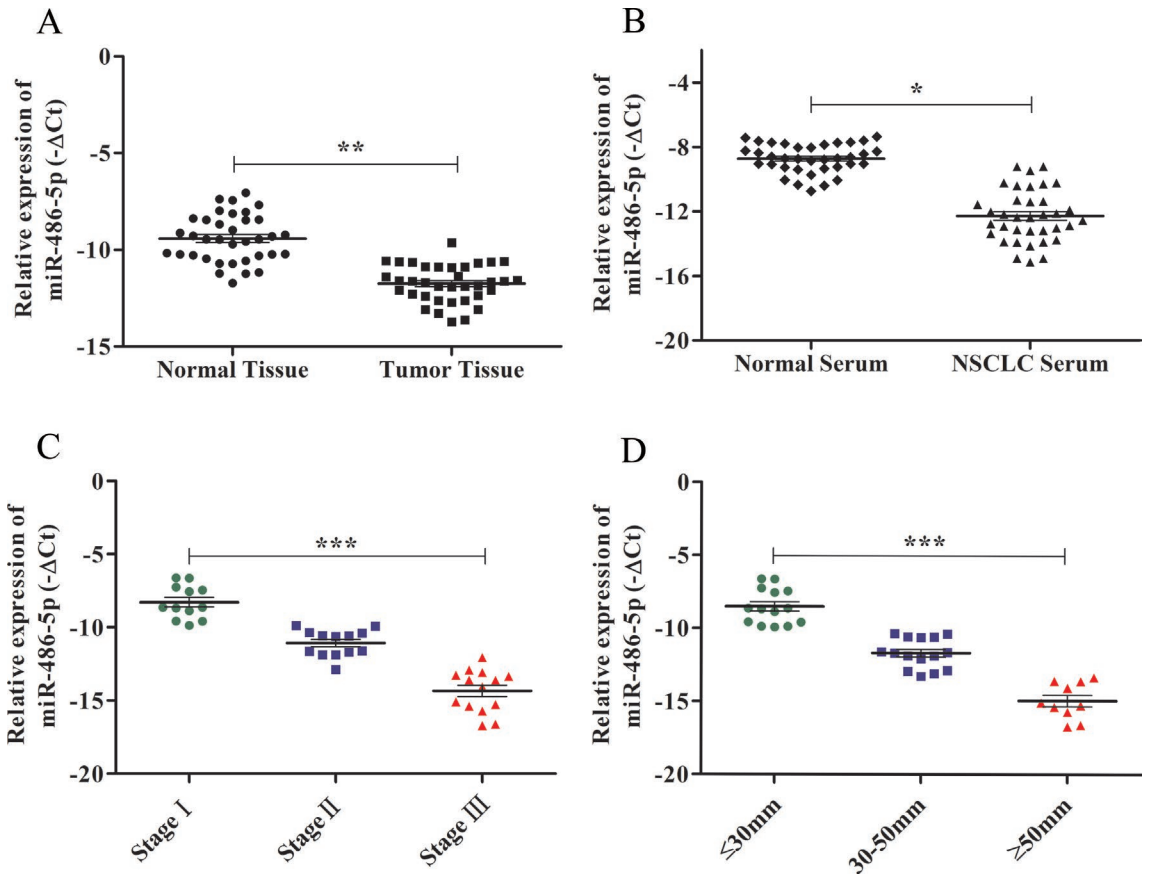

FIGURE 1 | The expression levels of miR-486-5p and correlations with clinical data in NSCLC samples by qRT-PCR. **(A)** The relative expression levels of miR-486-5p were calculated by −ΔCt in corresponding tumor tissue and normal tissue. **(B)** The relative expression levels of miR-486-5p in NSCLC patients' serum and normal serum by −ΔCt method. **(C)** The relative expression levels of miR-486-5p were significantly lower in stages II and III compared to stage I ($p < 0.001$). **(D)** The expression levels of miR-486-5p were significantly lower in the larger tumor ($p < 0.001$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

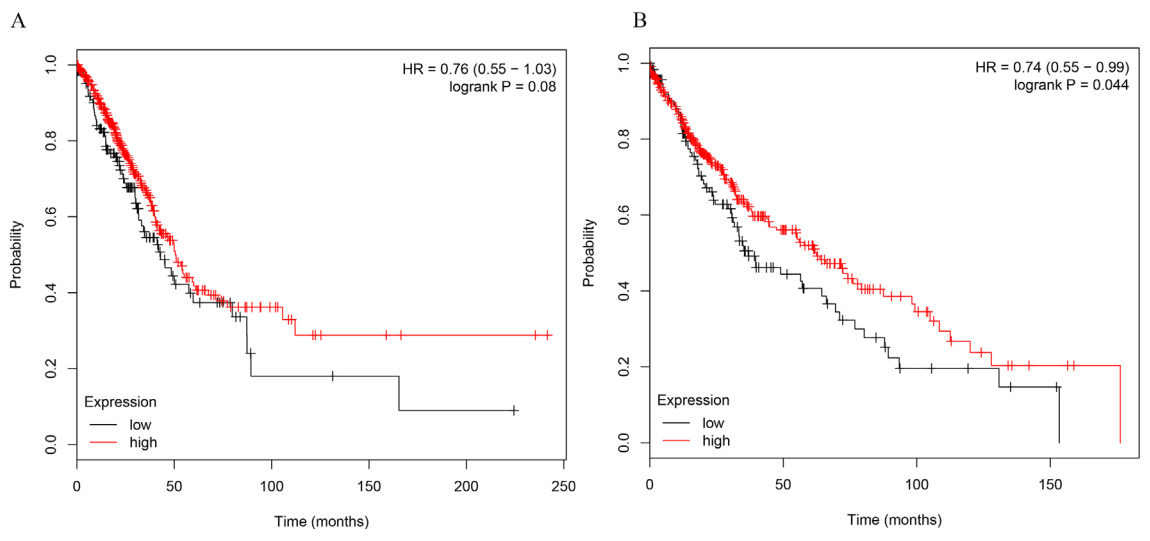

FIGURE 2 | The correlations of miR-486-5p levels and survival months in NSCLC patients. **(A)** No significant correlation between miR-486-5p expression and prognosis data in AC patients (504 samples, $p = 0.08$). **(B)** SCC patients with low expression of miR-486-5p had worse prognosis (472 samples, $p = 0.044$).

PIK3R1 Was a Direct Target of miR-486-5p

To elucidate the biological mechanism responsible for miR-486-5p as a tumor suppressor, bioinformatics analysis was adopted to search its target genes. Several potential targets of miR-486-5p were identified using microT, miRmap, TargetScan, PicTar, and miRanda programs based on the starBase v3.0. Specifically, the CLIP data were adopted with strict stringency ≥5; the pan-cancer data with six cancer types and above five programs were employed to predict. Totally, three mRNAs were identified, including ST5, PIK3R1, and SRSF3. However, no related papers have reported the regulation of miR-486-5p to the gene ST5 or SRSF3. On the contrary, some studies found the relations of miR-486-5p and PIK3R1 in other cancers: colorectal cancer and hepatocellular carcinoma (Huang et al., 2015; Zhang et al., 2018). Interestingly, miR-486-5p was reported to have different effects on the target PIK3R1 in A549 cell lines (Peng et al., 2013; Gao et al., 2018). Therefore, we preferentially selected PIK3R1 as one potential target of miR-486-5p to validate their relations in lung cancer. In particular, the 3'-UTR of PIK3R1 mRNA perfectly harbored complementary sequences to the miR-486-5p seed sequence (**Figure 3A**).

To further determine whether PIK3R1 could be directly regulated by miR-486-5p, we performed dual luciferase reporter assay. After 24 and 48 h with transfection, the luciferase activity of PIK3R1-3'-UTR wild type was reduced by ~ 52% and ~68% in cells expressing miR-486-5p compared to those expressing mimic NC, respectively ($p < 0.01$). However, the luciferase activity did not reveal significant reduction in PIK3R1-3'-UTR mutant type by miR-486-5p. A positive control with binding sites of miR-486-5p showed good consistent result with the wild-type case (**Figures 3B, C**). Therefore, PIK3R1 was validated to be a direct target of miR-486-5p, and miR-486-5p could inhibit PIK3R1 by binding to its 3'-UTR sequence.

To further elucidate the relationship of miR-486-5p and PIK3R1 in clinical NSCLC specimens, qRT-PCR assay found that PIK3R1 was significantly upregulated in NSCLC tissue compared with normal tissue (**Figure 3D**). Moreover, there was a significantly inverse correlation between miR-486-5p and PIK3R1 expression levels in tumor tissue ($r = -0.774$, $p < 0.01$,

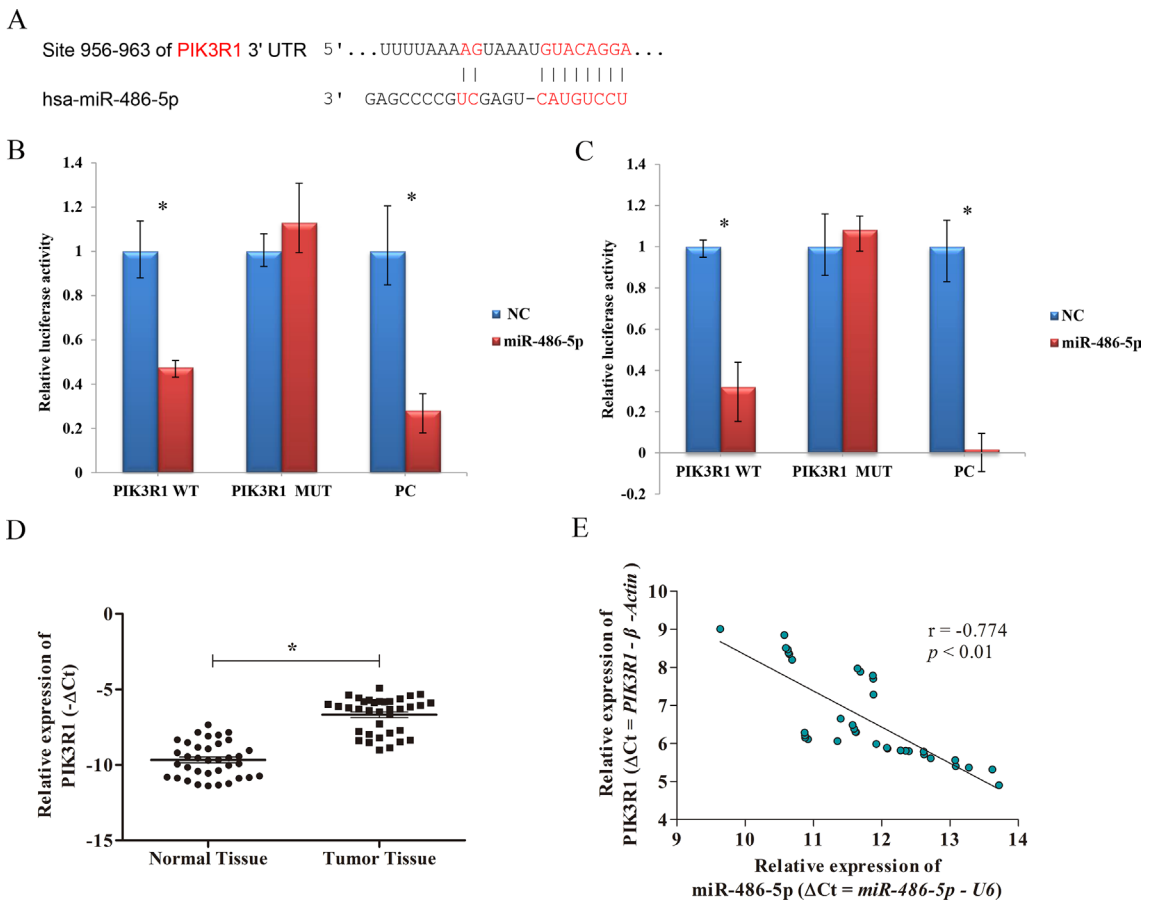

FIGURE 3 | The correlation of PIK3R1 and miR-486-5p expression levels. **(A)** PIK3R1 3'-UTR contains miR-486-5p binding sites. The alignment of the seed region of miR-486-5p and the site of target were indicated in red. **(B, C)** The luciferase reporter assay showed that the luciferase activity of PIK3R1-3'-UTR wild type (WT) was significantly reduced in cells expressing miR-486-5p compared with those expressing negative control (NC) ($p < 0.01$), but the luciferase activity of PIK3R1-3'-UTR mutation type (MUT) did not reveal significant changes after 24 h **(B)** and 48 h **(C)**. The positive control (PC) also exhibited reduction of luciferase activity after 24 and 48 h with transfection ($p < 0.01$). **(D)** The expression level of PIK3R1 was upregulated in NSCLC tumor tissue compared to the normal tissue ($p < 0.05$, $n = 36$). **(E)** Upregulation of PIK3R1 was negatively correlated with downregulation of miR-486-5p in NSCLC tissue by ΔCt ($r = -0.774$, $p < 0.01$, $n = 36$). *$p < 0.05$.

Figure 3E), it revealed that the expression of PIK3R1 might be affected by miR-486-5p in NSCLC.

Overexpressed miR-486-5p Inhibited PIK3R1 in NSCLC Cell Lines

We also found that miR-486-5p was downregulated in NSCLC A549 and H1299 cell lines compared to normal pulmonary epithelial cell lines, BEAS-2B (**Figure 4A**). Therefore, we hypothesized that miR-486-5p might function as a tumor suppressor. To validate that overexpressed miR-486-5p could lead to a reduction of PIK3R1 mRNA, pre-miR-486-5p lentivector was constructed and transfected into NSCLC A549 and H1299 cell lines. The results showed that miR-486-5p had observably higher expression in transfected A549 and H1299 cells than in the corresponding controls ($p < 0.01$) after 48 h, and the expression level of miR-486-5p was higher after 72 h incubation than 48 h in A549 cell line (**Figure 4B**) but not in H1299 (**Figure 4C**). Interestingly, the target PIK3R1 showed lower expression level in the transfected cells compared to the controls, especially after 72 h in A549 cells (**Figure 4D**, **E**). These results suggested that transfected cells could effectively upregulate miR-486-5p and inhibit PIK3R1 level.

To further determine whether the PIK3R1 protein was dysregulated by miR-486-5p, we transfected A549 cells with miR-486-5p (mimic) and two small interfering RNAs (siRNAs) specifically against PIK3R1 (si-739, si-1178) to inhibit PIK3R1 gene. As shown in **Figure 4F** by Western blot, miR-486-5p (~54% reduction), si-739 (~47%), and si-1178 (~61%) dramatically reduced the expression of PIK3R1 (p85α) after 48 h with transfection. Consistently, miR-486-5p could also effectively reduce the expression of PIK3R1 in H1299 cells (~57%). Therefore, the results revalidated that miR-486-5p effectively inhibited the translation of PIK3R1.

PIK3R1 Was Involved in miR-486-5p-Induced Suppression of NSCLC Cell Growth

As the overexpressed miR-486-5p inhibited PIK3R1, the phosphoinositide-3-kinase regulatory subunit 1, which could take part in the PI3K pathway, we sought to determine whether ectopic expression of miR-486-5p had effects on cell metabolism in NSCLC. Interestingly, CCK-8 proliferation assay revealed that forced expression of miR-486-5p significantly reduced cell

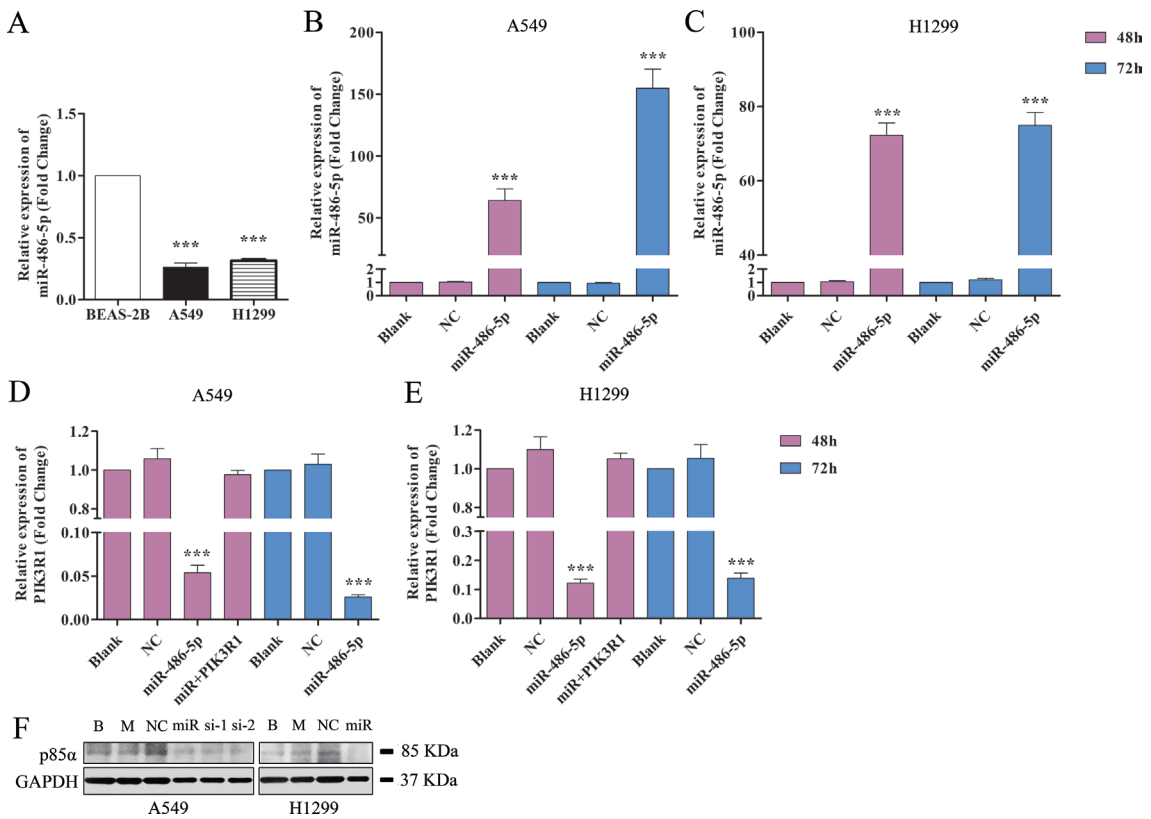

FIGURE 4 | Overexpressed miR-486-5p significantly inhibited the mRNA and protein levels of PIK3R1. **(A)** The fold change of miR-486-5p in A549 and H1299 cells compared to human normal pulmonary epithelial cell lines, BEAS-2B. **(B**, **C)** The relative expressions of miR-486-5p in transfected A549 **(B)** and H1299 **(C)** cell lines were significantly higher compared to the blank (with no transfection) and NC (negative control, transfected with empty vector) after 48 or 72 h with transfection. **(D**, **E)** Overexpression of miR-486-5p led to the reduction of PIK3R1 gene in A549 **(D)** and H1299 **(E)** cell lines after 48 or 72 h transfection. **(F)** The miR-486-5p mimic or two siRNAs dramatically reduced the protein expression of PIK3R1 compared with the controls in A549 or H1299 cells after 48 h transfection. ***$p < 0.001$, B: blank, M: mock, miR: miR-486-5p, si-1: si-739, si-2: si-1178.

growth compared to the blank control (with no transfection) and the NC (transfected with empty vector) in A549 (**Figure 5A**) and H1299 cell lines (**Figure 5B**) after 48 h ($p < 0.05$), especially after 72 h in A549 cells ($p < 0.01$). Moreover, si-PIK3R1 (si-1178) mimicked the effect of miR-486-5p overexpression. The blank and NCs had no significant difference; this suggested that cell proliferation was affected by miR-486-5p itself, not by the vector. To evaluate whether dysregulation of PIK3R1 was involved in cell proliferation as a target of miR-486-5p, A549 and H1299 cells were enforced to overexpress miR-486-5p and PIK3R1 at the same time. Compared to the cells with only transfection of miR-486-5p or blank, the cotransfected cells had significantly increased cell growth in both A549 (**Figure 5A**) and H1299 cell lines (**Figure 5B**). These observations suggested that the effects of miR-486-5p on the inhibition of cancer cell proliferation could be diminished by overexpressed PIK3R1. Consistently, the cotransfected cells increased the mRNA levels of PIK3R1 compared to the only overexpressed miR-486-5p cells in both A549 (**Figure 4D**) and H1299 cell lines (**Figure 4E**) after 48 h. In conclusion, miR-486-5p effectively inhibited cell proliferation by targeting PIK3R1.

To further explore cell invasion abilities with miR-486-5p overexpression, Transwell assay was performed and found that increased miR-486-5p could effectively suppress the migration and invasion of A549 (**Figures 6A**, **B**) and H1299 cell lines

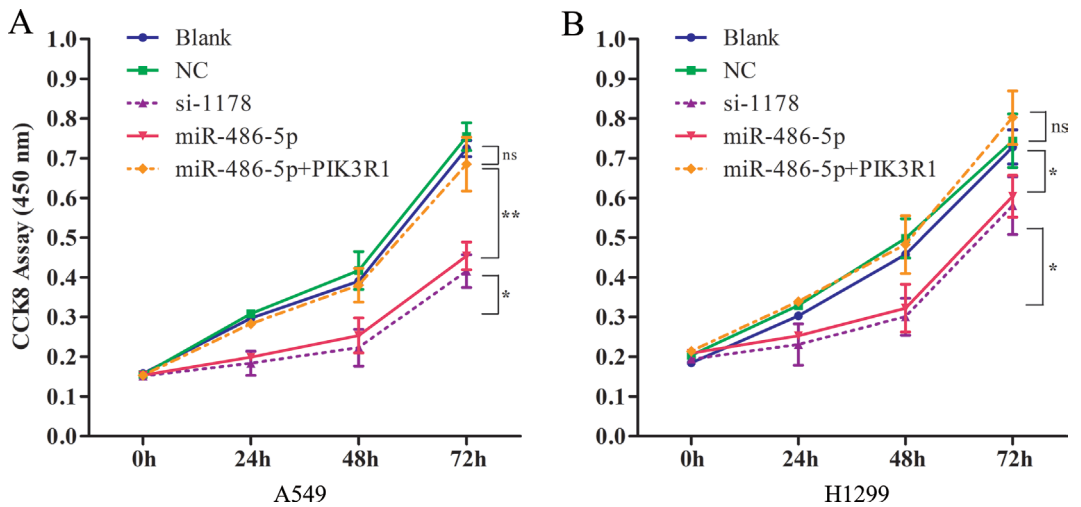

FIGURE 5 | PIK3R1 was involved in miR-486-5p-induced suppression of NSCLC cell proliferation. **(A)** The cell proliferation of A549 cells was evaluated with CCK8 assay with transfecting pre-miR-486-5p, si-1178, or pre-miR-486-5p plus PIK3R1 in 0, 24, 48, and 72 h. The absorbance was measured at 450 nm. The cell proliferation level of miR-486-5p or si-1178 group was significantly lower than the blank or NC after 48 h ($p < 0.05$) and extremely significantly lower after 72 h ($p < 0.01$). However, the pre-miR-486-5p plus PIK3R1 group diminished the effect (ns). **(B)** Similar situation of the cell proliferation in H1299 cell lines with overexpression miR-486-5p and PIK3R1. *$p < 0.05$, **$p < 0.01$, ns, not significant.

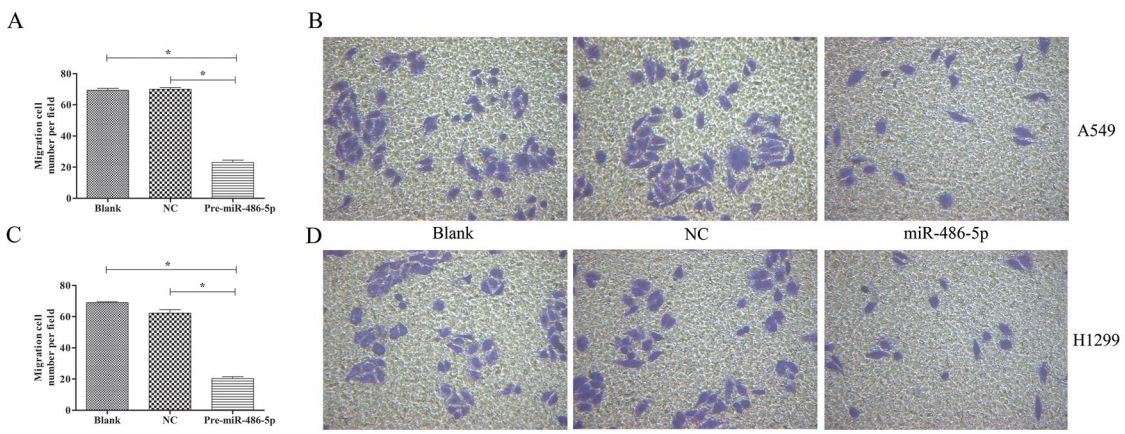

FIGURE 6 | Overexpressed miR-486-5p significantly inhibited A549 and H1299 cell invasion. **(A)** The migrated cell numbers of A549 cells were significantly reduced after 72 h incubation by Transwell assay. The number of cells was calculated with crystal violet staining. **(B)** Representative pictures of Transwell assay in A549 cell lines. Similar results were obtained in three independent experiments. **(C)** The migrated cell numbers of H1299 cells after 48 h incubation. **(D)** Representative pictures of Transwell assay in H1299 cell lines. *$p < 0.05$.

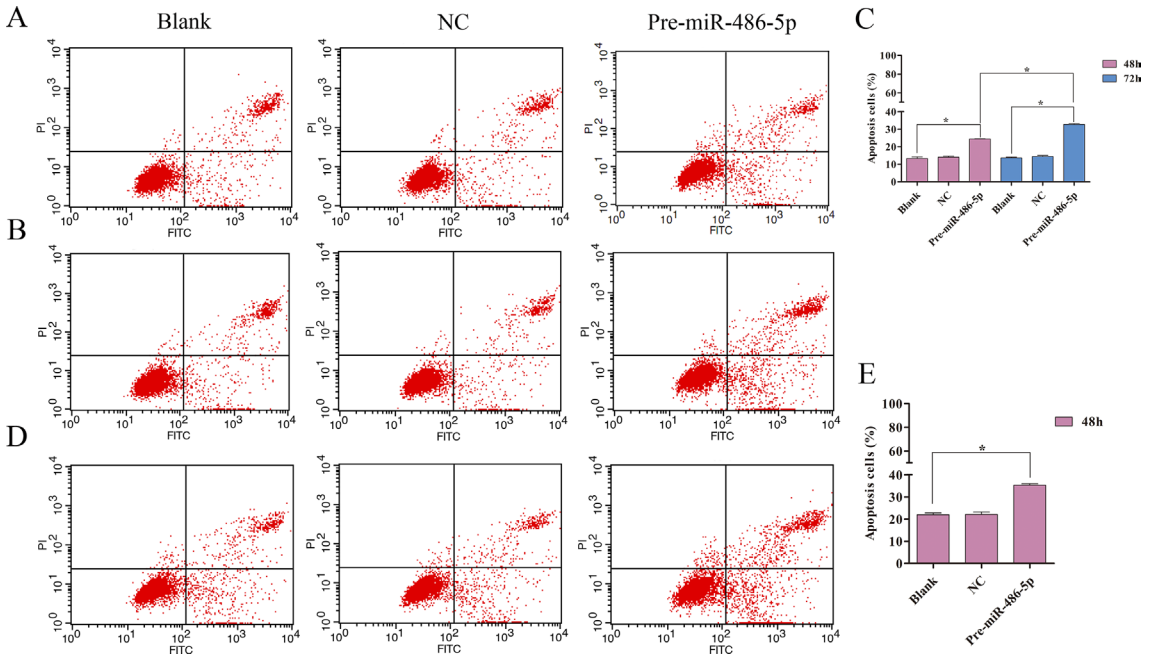

FIGURE 7 | Overexpressed miR-486-5p significantly induced A549 and H1299 cell apoptosis. **(A**, **B)** A549 cells were stained with annexin V fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) after 48 h **(A)** and 72 h **(B)** incubation. One representative experiment was shown, and similar results were obtained in three independent experiments. **(C)** The percentage of A549 apoptosis cells were shown after 48 and 72 h incubation, respectively. **(D)** One representative experiment was shown in H1299 cell apoptosis after 48 h with transfection. **(E)** The percentage of H1299 apoptosis cells were shown after 48 h incubation. *$p < 0.05$.

(**Figures 6C**, **D**) after 48 h. Furthermore, annexin V apoptosis assay showed that the pre-miR-486-5p case had a higher apoptosis rate compared with the controls in A549 (**Figures 7A–C**) and H1299 cell lines (**Figures 7D–E**), especially after 72 h in A549 cells (**Figure 7B**). The empty vector control (NC) did not reveal induced apoptosis on cells, which also validated that the effects were from the overexpressed miR-486-5p, not by the vector.

DISCUSSION

Our previous study demonstrated that miR-486-5p was significantly downregulated both in tissue and serum samples of NSCLC; it revealed that miR-486-5p might function as a tumor suppressor (Tian et al., 2016). We further validated that miR-486-5p was downregulated in additional 36 paired tissue and serum samples. Moreover, a lower level of miR-486-5p was found in NSCLC A549 and H1299 cell lines compared to normal pulmonary epithelial cell lines. MiR-486-5p is located on chromosome 8p11.21, which is one of the most frequent genomic deletion regions containing potential tumor-suppressive genes in various tumors (Oh et al., 2011). Allelic loss of the genomic region may be responsible for the downregulation of miR-486-5p. Furthermore, miR-486-5p is located in a CpG island on chromosome 2q35; epigenetic silencing by DNA methylation modification may also lead to miR-486-5p downregulation (Wang et al., 2014). Interestingly, some papers had reported the role of miR-486-5p on diagnosis (Yu et al., 2010; Tan et al., 2011; Jin et al., 2017; Sromek et al., 2017) and prognosis (Hu et al., 2010) in lung cancer. Nevertheless, the opposite expression levels and roles of miR-486-5p in lung cancer were found in these related reports. Therefore, we intended to further validate the effects of aberrant miR-486-5p on cell growth and the regulatory pathway in NSCLC. Moreover, several studies have shown that miR-486-5p had important functions by targeting different genes in NSCLC. Pang et al. (2014) validated that downregulated miR-486-5p and upregulated eIF4E led to the overexpression of Pim-1 kinase, a critical survival signaling factor in NSCLC. Peng et al. (2013) recently reported that miR-486 directly targeted insulin growth factor signaling and functioned as a potent tumor suppressor of lung cancer both *in vitro* and *in vivo*. In this study, PIK3R1 was identified as a target of miR-486-5p, and we intended to further evaluate whether PIK3R1 was involved in the suppression of miR-486-5p on NSCLC.

The PIK3R1 gene is localized on 5q13.1. PIK3R1 gene (phosphoinositide-3-kinase regulatory subunit 1) encodes the 85-kD regulatory subunit p85α, a well-accepted subunit of class IA PI3K (Zhao and Vogt, 2008; Mellor et al., 2012). PIK3R1 is the 11th most commonly mutated gene across cancer lineages in TCGA database (Cerami et al., 2012). Although several studies have shown that the expression of PIK3R1 gene is decreased or lost in human cancers (Taniguchi et al., 2010; Urick et al., 2011; Cizkova et al., 2013), few researches explore the PIK3R1 gene in lung cancer. On the other hand, studies have demonstrated that PIK3R1 abrogation might reduce tumor proliferation and migration (Weber et al., 2011; Peng et al., 2013; Huang et al., 2015; Yan et al., 2016). This indicates that PIK3R1 gene may

have different functions in diverse cancers. Therefore, cancer is an extremely complex disease and regulated by a lot of elements and biological processes. In this study, PIK3R1 was found to be upregulated as a potential oncogene in NSCLC.

Although miR-486-5p and PIK3R1 are respectively studied in various cancers, few studies investigate the regulative roles of miR-486-5p and PIK3R1 in lung cancer. However, miR-486-5p was reported to have different effects on the target PIK3R1 in A549 cell lines (Peng et al., 2013; Gao et al., 2018). Indeed, we identified several potential targets of miR-486-5p, including ST5 and SRSF3. The NCBI database shows that ST5 has the ability to suppress the tumorigenicity of Hela cells in nude mice. Although we selected PIK3R1 as a target to study here, there is no denying that the antioncogenic properties of miR-486-5p might not solely be explained by its ability to regulate a single gene because one miRNA could regulate numerous different genes in tumorigenesis. At the same time, single mRNA target could be also affected by numerous regulatory factors, including different miRNAs. Future studies are needed to identify and validate other targets of miR-486-5p. It will allow us to have deeper understanding underlying the cellular mechanism of development and progression in NSCLC.

Based on the results, it could be concluded that miR-486-5p might be involved in the *PI3K-Akt signaling pathway* by inhibiting PIK3R1 (**Supplementary Figure 2**). PI3K (PIK3CA and PIK3R1) produced PIP3 and eventually affected proliferation and antiapoptosis process (**Supplementary Figure 2**). Interestingly, the *PI3K-Akt signaling pathway* was involved in the *nonsmall cell lung cancer pathway* from the KEGG database (https://www.kegg.jp/). Moreover, EGFR and K-ras gene could affect PI3K gene in antiapoptosis, and PI3K might have an indirect effect on cell cycle and apoptosis in NSCLC. Therefore, the relations between miR-486-5p and PIK3R1 could be well applied in the *PI3K-Akt signaling pathway* and *nonsmall cell lung cancer pathway*.

In conclusion, our results revealed that miR-486-5p was significantly decreased in NSCLC tissue, serum, and cell samples as a diagnostic biomarker. Downregulated miR-486-5p was correlated with advanced stage and large tumor size in NSCLC, and low expression of miR-486-5p had significantly worse prognosis in lung SCC patients. The downregulation of miR-486-5p was negatively correlated with the upregulation of PIK3R1, a direct target gene, and overexpressed miR-486-5p inhibited cell proliferation and invasion in NSCLC. Overall, the results indicated that miR-486-5p might function as a diagnostic and prognostic biomarker, and a tumor suppressor in NSCLC therapy, and the findings could help us to better understand the mechanism of miRNA-related oncogenesis in lung cancer.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Regulations and Guidelines on Ethical Review of Biomedical Clinical Research (trials) from the Ethics Committee of Jiangsu Province People's Hospital. The protocol was approved by the Jiangsu Province People's Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FT and YS performed the assay. TO and NL performed the computations and data analysis. FT and QG wrote the manuscript. JW and JL provided the clinical and cell samples. YB, XX, and QG conceived and designed the study.

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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.2019.00688/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | The TCGA data showed miR-486-5p was significantly lower expressed in lung SCC (A, 322 samples, $p < 0.01$) and AC tissue (B, 430 samples, $p < 0.01$) compared to the controls.

SUPPLEMENTARY FIGURE 2 | The PI3K-Akt signaling pathway and the regulatory relations of miR-486-5p and PIK3R1. The red line indicated the enhanced process or gene expression, and the green line indicated the attenuated process or inhibition. The dashed indicated the possible indirect effect on antiapoptosis, proliferation and metastasis.

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REVIEW
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Circulating MicroRNAs in Cancer: Potential and Challenge

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MicroRNAs (miRNAs) are endogenous non-coding small RNA molecules that can be secreted into the circulation and exist in remarkably stable forms. Like intercellular miRNAs, circulating miRNAs participate in numerous regulations of biological process and expressed aberrantly under abnormal or pathological status. The quality and quantity changes of circulating miRNAs are associated with the initiation and progression of cancer and can be easily detected by basic molecular biology techniques. Consequently, considerable effort has been devoted to identify suitable extracellular miRNAs for noninvasive biomarkers in cancer. However, several challenges need to be overcome before the practical application. In this review, we discuss several issues of circulating miRNAs: biological function and basic transport carriers; extracellular cell communication process; roles as reliable cancer biomarkers and usage in targeted cancer therapy; and challenges for clinical application.

Keywords: miRNAs, circulating, cancer, biomarker, communication, therapy, challenge

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INTRODUCTION

MicroRNA (miRNA) was first discovered as the product of the *lin-4* gene in *Caenorhabditis elegans* in 1993 (Lee et al., 1993; Wightman et al., 1993). The small non-coding RNAs (19-22nt) develop post-transcriptional regulation by mRNA cleavage or translation repression, which depended on the complementarity degree of miRNA-mRNA. mRNA cleavage occurs when there is a perfect match, whereas imperfect combination results in gene repression (Bartel, 2004). A large number of studies have confirmed the role of microRNAs in various cancer-associated biological processes, such as proliferation, differentiation, apoptosis, metabolism, invasion, metastasis, and drug resistance. The pathological origin of cancer has also been proven to be directly related to the dysregulation of miRNAs. Moreover, miRNAs are tissue-specific. Different tumors have distinctive miRNA expression profiles. So far, the basic biogenesis and function of the intracellular miRNAs have been reviewed in a number of contexts. On the other hand, the presence of extracellular RNAs in serum/plasma was described first by Bartel (Bartel, 2004; Kibel, 2009) and various miRNAs are proved to exist in a stable cell-free form in body fluids and other extracellular environments, including plasma, serum, urine, saliva, seminal, ascites, amniotic pleural effusions, and cerebrospinal fluid (Weber et al., 2010; Cortez et al., 2011; Husted et al., 2011; Alečković and Kang, 2015; Kibel, 2009). Studies suggested that they are injected to the circulation in different ways. Parts are due to the passive leakage of apoptosis, necrosis, or the environment of inflammation, and parts are secreted actively by exosomes/microvesicles, lipoproteins, and RNA-protein complex (Arroyo et al., 2011; Cheng, 2015). Furthermore, specific miRNAs are selected to pack into exosomes and become one of the most important aspects of the tumor microenvironment, which is the

underlying mechanism of tissue/disease specificity of circulating miRNAs. Additionally, circulating miRNAs are correlated with the degree of tumor progression and present differently at different stages of cancer, making them play an important role in cancer immunotherapy. That is to say, the presence of certain type of circulating miRNAs was confirmed essentially in the manifestation, development, invasion, and metastasis of cancer, and the abnormal levels of distinct miRNAs could be observed in every process above (Wang et al., 2018). Although the cancer-related biomarkers, which are widely used clinically, are simple and fast, their disadvantages, like poor early diagnosis and prognostic value, limit their role in targeted therapy and the lack of tissue specificity leads to an urgent need to find novel biomarkers. As a result, circulating miRNAs are becoming candidates of emerging non-invasive cellular and molecular biomarkers of cancer.

BIOLOGICAL FUNCTION AND CARRIER PROFILES OF CIRCULATING MIRNAS

The discovery of circulating miRNA is unanticipated, considering that the activity of RNase in plasma and the underlying mechanism came to be known after years of intensive experiments and discussion. In 2008, Patrick and colleagues confirmed that endogenous miRNAs ranging 18 to 24 nt exist in human plasma by cloning, sequencing, and quantification. Storing at room temperature, suffering freeze-thaw, or extreme variations in pH will not lead to a descending of circulating miRNAs (Duttagupta et al., 2011; Kibel, 2009). However, purified plasma (Arroyo et al., 2011) miRNAs and synthetic miRNAs (Tsui et al., 2002; Kibel, 2009) rapidly degraded when cultured with plasma, suggesting that endogenous miRNAs are resisted to RNase because of various modifications. There are two major populations of circulating miRNAs, vesicle-associated and non–vesicle-associated (**Figure 1**), which reflect the different mechanisms of release. Some tissue-specific miRNAs may release to the circulation in a protein-mediated way and thus presented in protein complex only, such as the liver-specific miR-122 (Chang et al., 2004). However, because releasing vesicles are essential in the process of the maturation and activation of most blood cells, almost all erythrocytes and platelet-related miRNAs are packed into the vesicles in the circulation (Heijnen et al., 1999; Hunter et al., 2010).

Membrane-bound vesicles, such as exosomes (50–90 nm) and microvesicles (1 μm), comprise one type of extracellular miRNA,

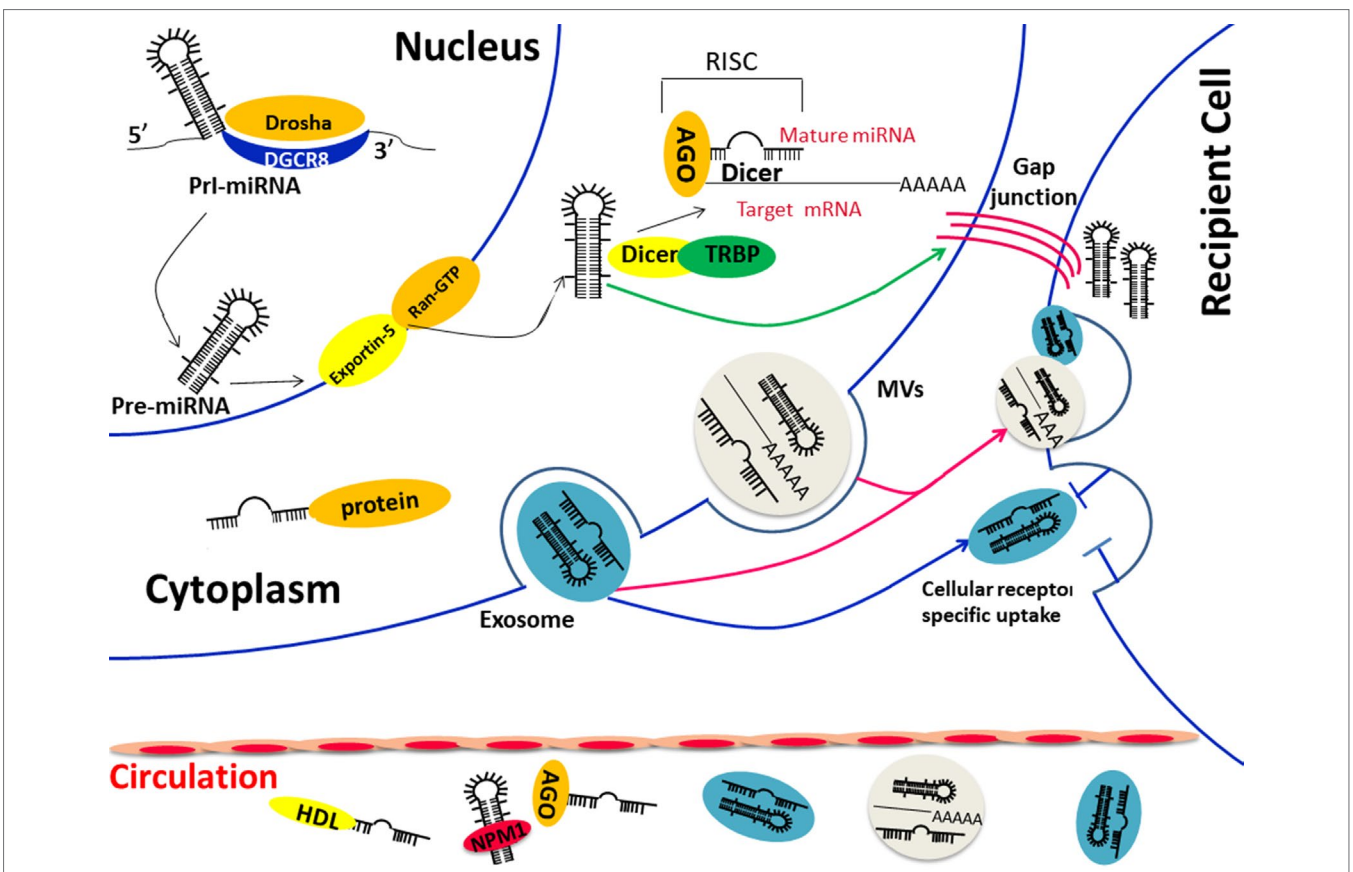

FIGURE 1 | (1) Biological functions and transportation carriers of circulating miRNA; (2) Diverse ways of miRNAs in cell communication: direct fusion and endocytosis of extracellular vesicles (exosomes orMVs) (red arrow) or direct transfer through cell gap junction (green arrow) or the indirect identification of specific surface receptor (blue arrow). Abbreviations: Pre-miRNA, precursor miRNA; Pri-miRNA, primary miRNA; AGO, Argonaute; TRBP, transactivation-responsive RNA-binding protein; DGCR8, DiGeorge Syndrome Critical Region Gene 8; MVBs, multivesicular bodies; NPM1, nucleophosmin 1.

which can be detected from vesicles isolated and purified from plasma/serum (Valadi et al., 2007; Taylor and Gercel-Taylor, 2008; Hunter et al., 2010). A study done by a team from America found multiple heterogeneous RNAs via Bioanalyzer from exosomes derived from a mast cell line. Large amounts of small RNA were found while the level of ribosomal RNA was low. Then, more than 120 miRNAs were certified in further analysis by microarray (Valadi et al., 2007). There is no doubt that the formation of the miRNA-vesicle package is confirmed by lots of researches. However, recent studies found that the apoptotic body is also one of the forms of the vesicular carrier, but the miRNA it contains may be a random event that differs from the exosome. In a word, all these results indicated the function of MVBs as transporters for extracellular miRNAs.

On the other hand, other studies confirmed that majority of the circulating miRNAs exist in a non–vesicle-associated form, such as the ribonucleoprotein complex. The copies of miRNAs dropped significantly since proteinase K was added to the plasma, which verifies the hypothesis that miRNAs could be degraded by RNase easily when dissociated from a protease-sensitive complex(10), and these proteins, such as Argonaute2 (Ago), GW182, nucleophosmin1 (NPM1), and high-density lipoprotein (HDL), are confirmed as miRNA carriers in a large number of studies. Ago2, the central protein of miRNA-mediated interference, together with GW182, were verified responsible for the protection and transport of extracellular miRNAs (Wang et al., 2010; Arroyo et al., 2011; Yao et al., 2012; Montani and Bianchi, 2016). The miRNA degradation occurred when Ago2 was isolated from the protein complex (Arroyo et al., 2011) and when GW182 was knocked down (Yao et al., 2012). Another protein that takes part in the protection of external miRNAs is the well-known nucleolar RNA-binding protein, nucleophosmin 1 (NPM1), which is involved in the exporting of RNAs and ribosome (Maggi et al., 2008). Synthetic miR-122 was not degraded by RNase when incubated with NPM1, and the NPM1 was confirmed not only participated in the packaging of miRNAs but also in the process of miRNA exporting (Wang et al., 2010). High-density lipoprotein is widely known as a mediation of excess cellular cholesterol. However, its function is far more complex especially when it involves the transportation and post-transcription effect in the recipient cells of miRNAs (Rothblat and Phillips, 2010; Vickers et al., 2014). As a result, HDL has been used as the delivery of siRNAs in animal models (Kim et al., 2007).

In conclusion, it is just these miRNA transporters that protect circulating miRNAs from RNase in various body fluids. It may be associated with the cell type and tissue specificity, which carrier miRNAs "select" and the carrier of one specific miRNA may be not unique (Arroyo et al., 2011; Li et al., 2012).

CIRCULATING MiRNAs IN CELL COMMUNICATION

Traditional cell–cell communication means gap junctions or cell signal transduction, such as neurotransmitter, hormone, and cytokines, whereas the extracellular miRNA-dependent cell-cell communication is proven to be induced by membrane-derived vesicles recently (Xu et al., 2013). Exosomes were demonstrated to be related to the immune function many years ago, and researchers were surprised to confirm that both mRNA and miRNAs could be packaged to the "magical" vesicles and are exported or released from cells in response to biological stimuli (Cortez et al., 2011). The lipid bilayer is the coat of exosome that reflects the information from the original cell, and the proteins on the surface are important in cell signal pathway (Zhao et al., 2015). Respiratory chain inhibitor rotenone was able to reduce the level of extracellular miRNAs (Wang et al., 2010), and the mRNA and miRNA profiles of exosomes are different from those of the parent cells (Valadi et al., 2007), suggesting that the secretion of exosomes is an ATP-dependent and active-selecting process. On the other hand, the difference between the spectra of intracellular and extracellular miRNAs under the condition of no cytolysis indicated that the cellular control mechanism was involved in the release of extracellular miRNAs(20). In *in vitro* experiments, many miRNAs were overexpressed in the culture medium after the intervention of serum starvation and proved to be related to the cell cycle arrest, apoptosis, and cell death, suggesting that the biological function of miRNAs may extend outside of the cell and mediate cell–cell communication (Wang et al., 2010). A few selected miRNAs showed a trend of translating from intracellular to extracellular during the first 2 h of serum elimination, which suggested that there is a process of prepackage and storage of miRNAs (Wang et al., 2010). Those encapsulated miRNAs are able to reach the remote area and affect recipient cells, especially various immune cells in the tumor microenvironment, which is important for tumorigenesis (Gong et al., 2012; Lässer, 2012). miRNAs could participate in a directional transfer from T cells to antigen-presenting cells using exosomes as a carrier. Not surprisingly, miRNAs in exosomes are induced by immune cells different from their parent cells, indicating that miRNAs were changed in the process of intercellular communication during immune interactions (Mittelbrunn et al., 2011). Other immune cells, such as dendritic cells (DCs), were proven to transfer signals to neighboring DCs via exosome shuttle miRNAs, and the packed miRNAs were different according to the maturation of the DCs (Montecalvo et al., 2012). That is to say, exosomal miRNAs are one of the complex strategies of immune cell communication. Although the exact underlying mechanism of exosomal miRNAs in cell communication is still unclear now, we have obtained evidence of several hypotheses (**Figure 1**): 1) direct fusion of vesicles and receptor cell membranes (Mulcahy et al., 2014), 2) active endocytosis or phagocytosis by receptor cells, 3) identification of proteins on the surface of exosomes and receptors of recipient cells (Munich et al., 2012; Corrado et al., 2014), 4) cell gap junction-mediated transfer (Lim et al., 2011; Aucher et al., 2013). The communication between circulating miRNAs and target cells will lead to a series of effects on both physiological and pathological conditions (Kosaka et al., 2010; Pegtel and Kieff, 2010) and the packed miRNAs make an exchange of genetic material additionally (Valadi et al., 2007). Some miRNAs exist in exosomes not derived from their parental cell. These have confirmed that the gene transfer is mediated by exosomes (Valadi et al., 2007; Zhang et al., 2015a). The extracellular

miRNAs not only affect the surrounding cells but also the distant tissues, thereby leading to the progression of diseases (Mathivanan et al., 2012).

Adipose tissue contains a different type of circulating exosomal miRNA that can regulate distant cells as a form of adipokine, which is confirmed by the experiment that miRNAs expressed in the brown adipose in one mouse could regulate its target liver reporter in other mouse (Thomou et al., 2017). Additionally, accumulating evidence has shown that circulating miRNAs participate in the invasion and metastasis of cancer via cell communication with recipient cells (Mei et al., 2011). Secreted miRNAs from metastatic cells were transported to endothelial cells and promoted angiogenesis, which are regulated by neutral sphingomyelinase 2 (Kosaka et al., 2013a). All of these studies indicate that (Lee et al., 1993) sending information via extracellular miRNA is another way of intercellular communication (Wightman et al., 1993), miRNA-dependent cell–cell communication is the best explanation for the existence of circulating miRNA, and (Bartel, 2004) circulating miRNAs secreted by cancer cells can trigger tumorigenesis in the recipient cells.

CIRCULATING MiRNAs AS PROMISING CANCER BIOMARKERS

miRNAs are a family of endogenous 19-22nt noncoding RNAs involved in posttranscriptional regulation and participate in various physiological and pathological processes by inhibition of translation or by mRNA cleavage. More than 50% of protein-coding genes are assumed to be the target of miRNAs (Krol et al., 2010). The miRNA expression is frequently dysregulated in cancer, forming tissue-specific expression patterns. In addition to their intracellular biology functions, numerous studies have documented that the dysregulation of extracellular miRNAs is associated with the origin, progression, therapeutic response, and patient survival of the disease since the presence of circulating miRNAs in serum was first described (Wang et al., 2018; Zhang et al., 2018). Mouse prostate cancer xenograft model was used to identify whether the abnormal expressed serum miRNA was tumor-derived in *in vivo* experiment. Results showed that the levels of miR-620 and miR-629 in the serum were different and can distinguish cancer-bearing mice from controls (Mitchell et al., 2008). There was a surprisingly distinct miRNA expression between endothelial cells cultured with cancer cell lines with control, which means the upregulated miRNAs were induced by tumor cells (Zhuang et al., 2013). At the same time, this study confirmed the hypothesis that stimulated miRNAs were packed into microvesicles and delivered to endothelial cells in follow-up experiments (Skog et al., 2008; Zhuang et al., 2013). On the other hand, the phenomenon that tumor-suppressing miRNAs were amplified, whereas oncogenic miRNAs were reduced, reflected the fact that circulating miRNAs were not the primary products of cancer cells, but the results of global immune response and play an important role in cancer defense and cancer therapy (Aucher et al., 2013). However, another phenomenon is that cancer cells transfer the intercellular tumor-suppressive miRNAs to the extracellular environment, modify tumor microenvironment, and support cancer progression. In other words, extracellular miRNAs can act as both oncomir and suppressor by different stimuli. These studies provided the evidence that miRNAs may derive from tumor cells in response to specific signals, enter the circulation in a stable form as cancer-related molecules, and contribute to early diagnosis, prognosis, and individualizing therapeutic strategies (**Table 1** (Gonzales et al., 2011; Silva et al., 2011; Zhou et al., 2011; Bryant et al., 2012; Sun et al., 2012; Valladares-Ayerbes et al., 2012; Kawaguchi et al., 2013; Ng et al., 2013; Nguyen et al., 2013; Takeshita et al., 2013; Tanaka et al., 2013; Wang et al., 2013; Zeng et al., 2013; Enache et al., 2014; Geng et al., 2014; Ogata-Kawata et al., 2014; Zanutto et al., 2014; Zhang et al., 2014; Chen et al., 2014a; Wang et al., 2014; Antolin et al., 2015; Stuckrath et al., 2015; Zhang et al., 2015b; Zhang et al., 2016; Mirzaei H. et al., 2016; Mirzaei H. R. et al., 2016; Zhang et al., 2017; Zhao et al., 2017; Bahrami et al., 2018; Jamali et al., 2018; Shekari et al., 2018; Wang et al., 2018).

Circulating MiRNAs as Biomarkers for Early Diagnosis

The diagnosis of cancer currently suffers from low sensitivity, because many tumors cannot be found at the early stage and delay the treatment until it is too late. MiRNA expression is frequently dysregulated in cancer, forming a particular expression profile and, thus, benefits the early detection of cancer. MiRNAs associated with tumor growth are highly expressed, whereas suppressors are expressed lower. Therefore, these tissue-specific miRNAs are becoming emerging candidates in cancer diagnosis. Selected plasma/serum circulating miRNAs could be used to discriminate various cancer patients from healthy individuals such as breast (Antolin et al., 2015), colorectal (Zanutto et al., 2014), gastric (Zhang et al., 2015b), lung (Zhao et al., 2017), pancreatic (Kawaguchi et al., 2013), and hepatocellular (Mirzaei H. R. et al., 2016) cancers, making them tools for earlier diagnosis. In addition, differential concentrations of miRNAs were detected among different cancer subtypes and differentiation grades in breast cancer; aberrant levels of miRNAs were associated with the HER2 and estrogen receptor status as well, indicating the diagnostic and therapy-selecting potential of circulating miRNAs (Stuckrath et al., 2015). The expression of miR-21 was associated with the clinical stage and molecular subgroup of diffuse large B-cell lymphoma (DLBCL), which means that patients in early stage have a higher concentration of serum miR-21 than those in stage III and IV and patients with different subgroup have an obvious differentiation (Chen et al., 2014b). On the other hand, miRNAs show a remarkable relationship with tumor derivation, which is important in the identification of metastatic tumors with unknown primary origin. A microarray of 48 selected miRNAs could trace and classify 90% primary tumor in metastatic samples (Rosenfeld et al., 2008). The combination of miR-145 and miR-451 could discriminate breast cancer from healthy individuals as well as other types of cancers, including liver cancer, lung cancer, and colorectal cancer, which validated the function of circulating miRNAs in cancer classification (Ng et al., 2013). Furthermore, not only cancers could be distinguished from normal ones but also those who suffered from chronic inflammation were

TABLE 1 | Values of circulating miRNAs in different cancers.

Cancers	Expression profile and treatment value	Diagnostic value	Prognostic value	Diagnostic and prognostic value	RE
Gastric cancer	upregulated	miR-421 miR-20a miR-103 miR-181c miR-378 miR-221 miR-744 miR-192 miR-199a-3p miR-486-5p miR-199a-3p miR-423-5p miR-107 miR-194 miR-17 miR-1 miR-34a miR-27a miR-185 miR-210 miR-148a miR-146a miR-218 miR-18a miR-214 miR-301a miR-223 miR-16 miR-100 miR-451 miR-106a miR-222	miR-21 miR-25 miR-200c	52-56
	downregulated	miR-195-5p miR-17-5p let-7a miR-106b miR-375 miR-320a miR-218 miR-203 miR-93 miR-92b miR-19b-3p miR-16-5p	miR-196a miR-122	
Esophageal cancer	upregulated	miR-223-3p miR-192-5p miR-28-3p miR-223 miR-22 miR-21 miR-127-3p miR-296-5p miR-20b-5p miR-10a miR-100 miR-148b miR-133a miR-367 miR-200c	miR-1246 miR-146a	57-60
	downregulated	miR-100-5p miR-375 miRNA-718		
Pancreatic cancer	upregulated	miR-378* miR-409-3p miR-1290 miR-26a miR-18a miR-146b-3p miR-200a miR-200c miR-210 miR-221 miR-21 miR-194	miR-141 miR-375	61-63
	downregulated	let-7b-5p let-7c-5p miR-409-3p		
Breast cancer	upregulated	miR-195 miR-376c miR-409-3p miR-148b miR-299-5p miR-145 miR-191 miR-382 miR-215 miR-133a miR-133b miR-92a miR-192 miR-1 miR-411 miR-195 miR-202 miR-122 miR-141	miR-21 miR-375 miR-34a miR-125b miR-210 miR-801 miR-10b miR-155	64-68
	downregulated	miR-181a-5p miR-34 miR-92a miR-139-5p miR-143 miR-133a miR-30a let-7a miR-145 miR-365 miR-375 miR-30a miR-205 miR-342-5p miR-200c miR-497 let-7b	miR-768-3p	
	Therapeutic target	miR-155 (upregulated) miR-214 (downregulated)		
HCC	upregulated	miR-122 miR-801 miR-885-5p miR-192 miR-223 miR-130b miR-18 miR-15b miR-26a miR-27a miR-221 miR-1		69-71
	downregulated	miR-16 miR-199a miR-21		
Prostate cancer	upregulated	miR-378* miR-409-3p miR-1290 miR-26a miR-18a miR-146b-3p miR-210 miR-21 miR-221 miR-19 miR-200a miR-200c	miR-141 miR-375	72-74
	downregulated	let-7b-5p let-7c-5p	miR-409-3p	
NSCLC	upregulated	miR-20a-5p, miR-141-3p, miR-145-5p, miR-155-5p, miR-223-3pmiR-126-3p miR-210-3p miR-16-5p miR-182-5p, miR-183-5p, miR-320b miR-23b-3p miR-10b-3p miR-195-5p miR-4257-3p miR-222-3p	miR-21-5p 39 40 41 30 31	11 75-78
	downregulated	miR-198 miR-361-3p miR-625 let-7f miR-30e-3p		
	Therapeutic target	miR-181-5p miR-361-5p miR-205-5p miR-10b miR-30a-3p miR-30e-3p miR-15b miR-9-5p		
Colon cancer	upregulated	miR-27a-3p, miR-142-5p miR-409-3p miR-223 miR-92a miR-601 miR-760 miR-18a miR-1229 miR-1246 miR-150 let-7a miR-7 miR-93 miR-29a miR-23a miR-29c miR-200c miR-20a miR-130 miR-145 miR-216 miR-372 miR-378	miR-23a-3p miR-376c-3p mi-221 miR-141 miR-21	79-82
	downregulated	miR-125a-3p miR-34a miR-181b miR-92a miR-601 miR-760 miR-203 miR-31 miR-4772-3p		

singled out by a distinct miRNA expression pattern. Recent studies suggest that circulating miRNAs are involved in the regulation of inflammation, influence the genetic/epigenetic profile, and capable of predicting the unhealthy incidents (Olivieri et al., 2016). For example, a selected miRNA expression panel could differentiate pancreatic cancer from chronic pancreatitis with relatively high accuracy (Bloomston et al., 2007), whereas it can be used as biomarkers in the identification of hepatitis B virus (HBV) infection and HBV-positive hepatocellular cancer (Li et al., 2010). However, what needs to be pointed out is that the same miRNA can act as either oncogene or suppressor gene, depending on different cancer types (Cortez et al., 2011). MiR-125b could suppress cell proliferation and induce cell cycle arrest in ovarian, thyroid, and oral cancers (Visone et al., 2007; Nam et al., 2008), whereas it functioned oppositely in prostate cancer (Le et al., 2009). As a result, it is important to find out the corresponding abnormally expressed miRNAs in every type of cancers.

On the other hand, the phenomenon illustrated that some of the miRNAs are aberrantly expressed in tumors with an obvious familial aggregation tendency that can be applied to genetic diagnostics. MiR-15 and miR-16 are down-regulated in most of the B-cell chronic lymphocytic leukemia (B-CLL) patients due to the 30-kb region of loss in chromosome 13q14, which is the most frequently deleted genomic region of B-CLL (Calin et al., 2002). Similarly, acute myeloid leukemia patients with chromosomal translocations were proved together with a low level of miR-223 (Fazi et al., 2007). Even single nucleotide polymorphisms (SNPs) in miRNA genes may affect the biogenesis of miRNAs and thus increase the risk of cancer. SNP (rs417309), located in the 3′-UTR of DGCR8, was consistently associated with the possibility of suffering from breast cancer by the mechanism of interrupting the binding of miRNA, whereas an SNP in let-7 complementary sites could increase the risk of non-small cell lung cancer (Chin et al., 2008) as well. These results are also consistent with the studies that genes of miRNAs are most often located at fragile sites and genomic regions associated with cancers (Calin et al., 2004).

Circulating MiRNAs in the Prediction of Prognosis

A large number of studies have suggested the prognostic and predictive values of cancer-related circulating miRNAs as they participate in the regulation of the development of cancer. In the progression of cancer into a more invasive phenotype, miRNAs change as molecular labels of tumor cells, and the changes can be observed from tumorigenesis throughout the following progression. Therefore, circulating miRNAs are one of the most reliable candidates in disease monitoring. Circulating miR-142-3p correlated with a high risk of recurrence in lung adenocarcinoma patients of early stage (Kaduthanam et al., 2013). The levels of serum miR-155 could reflect the effect of surgery and chemotherapy in breast cancer, whereas the conventional biomarkers, such as carcinoembryonic antigen (CEA) and tissue polypeptide-specific antigen (TPS), were not that sensitive (Sun et al., 2012). Altered circulating miRNAs have also been proven to be bound up with the metastasis of cancer, and miR-141 achieved positive results in a test in prostate cancer patients in terms of identification of micro-metastasis (Gonzales et al., 2011). Decreasing levels of cir-miRNA-126 were related to treatment benefit in metastatic colorectal cancer, as it was proven to be associated with angiogenesis by way of paracrine (Hansen et al., 2015). Similarly, higher levels of circulating miR-122 have a positive correlation with the metastatic recurrence in stage II–III breast cancer patients (Wu et al., 2012). MiR-375 and miR-200b in the serum were significantly upregulated in patients with metastatic prostate cancer compared with patients with localized cancer (Bryant et al., 2012). Some of the other miRNAs that influence the epithelial phenotype of cancer cells were found elevated in the blood of gastric patients and induce invasion and migration (Valladares-Ayerbes et al., 2012). Additionally, circulating miR-214 and miR-373 were related to lymph node metastasis as well (Chen et al., 2013). Responsive miRNAs were observed valuable in therapy monitoring in head and neck squamous cell carcinoma (Summerer et al., 2013).

On the other hand, the changes of circulating miRNAs during chemotherapy and radiotherapy of cancer are well appreciated in many studies. Non-small-cell lung cancer (NSCLC) patients with clinical stage Ib to IIIa often need comprehensive treatment including operation and chemotherapy; a prediction for drug and chemotherapy sensitivity in advance can reduce the unnecessary toxic chemotherapy. A selected serum miRNA panel may serve as a predictor for the purpose of the above and found to be associated with the overall survival of NSCLC (Hu et al., 2010). Serum miR-125 and miR-22 led to the poor response to cisplatin-based and pemetrexed-based chemotherapy separately in NSCLC patients (Cui et al., 2013; Franchina et al., 2014). Serum miR-21 was associated with the relapse-free survival in DLBCL (Lawrie et al., 2008). MiR-150 was sensitive to acute radiation exposure and thus useful for the evaluation of treatment and toxic dose, which is essential for clinical radiation therapy (Jacob et al., 2013). All these studies suggested that circulating miRNAs are promising invasive biomarkers and are considered to be valuable in tumor classification, treatment strategy selection, cancer prognostication, and monitoring.

Circulating MiRNA-Based Cancer Therapy

Currently, more and more studies focus on the biological behavior of circulating miRNAs. It was found that not only mRNAs and proteins that are packed into the MVs or exosomes, but also miRNAs are proved to be existing in the MVs and exosomes abundantly. These exosomes secreted by donor cells containing packed miRNAs could be taken by recipient cells, both in the surrounding and remote area, which is the theoretical basis of miRNA-based targeted cancer therapy via vesicles. However, the delivery method of miRNA is an essential problem to resolve for RNAi therapy. Compared with carriers of targeted therapy, such as viruses, lipid, and polymeric nanoparticles, microvesicles serve as a natural carrier and could avoid attack from the immune system (Cheng, 2015), which is quite important in the persistence of drug intervention. Therefore, MVs and exosomes are used for the delivery of therapeutic RNAi as a more effective strategy in cancer therapy (Kosaka et al., 2013b). In other words, the transfer of synthesis tumor-suppressive miRNAs or antisense

of tumor oncogenesis miRNAs into target tumor cells through MVs or exosomes deserves continuing concern. MiR-150 is an immune-related miRNA and participates in the secreting of vascular endothelial growth factor via the regulation of tumor-associated macrophages and plays positive role in tumor growth. Experts transfer antisense miR-150 to MVs and inject "modified" MVs to mice via tail. Results showed that MVs could deliver the antagonucleotide for onco-mirna into tumor efficiently, and thus prevent tumorigenesis (Liu et al., 2013). Using GE11, a kind of peptide that can bind to the epidermal growth factor receptor, miR-let-7a reached the breast cancer tissue of xenograft mice model specifically and inhibited tumor development *in vivo* successfully (Ohno et al., 2013). MVs derived from human adult liver stem cells containing several anti-miRNAs could inhibit the growth of the tumor (Valentina et al., 2012). Furthermore, anti-mRNAs were also effective in drug resistance. For example, miR-9 was associated with the drug efflux transporter and was found upregulated in temozolomide-resistant glioblastoma multiforme (GBM) cells, whereas mesenchymal stem cells (MSCs)-derived exosomes complete the key biological processes of transferring anti-miR-9 from MSCs to GBM cells and reverse the chemoresistance finally (Munoz et al., 2013). Taken together, tumor-suppressive miRNAs could be delivered to target cancer cells *in vivo* and can be promising small RNAs for cancer therapy. However, the decomposition by the reticuloendothelial system and the inappropriate immune responses are the major issues of miRNA-relevant cancer therapy before their application.

CHALLENGES IN USING CIRCULATING MiRNAs AS CANCER BIOMARKERS

As we discussed above, circulating miRNAs are becoming potential non-invasive biomarkers for the prediction, prognosis, and therapeutic targets for cancers. Despite their many advantages, there are still challenges to overcome before clinical application.

Technical Challenges

The fundamental technical constraint to solve is the isolation and purification of samples, as the integrity and purity of RNA are the basic of detection and quantification. Unlike intercellular miRNAs, circulating miRNAs are interference by other components in serum easily and need to be cautious when centrifuged from serum (Cheng et al., 2013). It is necessary to add a step for purification as cell-free miRNAs are modified with exosomes, microvesicles, AGO2/NPM1, and HDL (Lee et al., 1993). Besides, the storage time and conditions also impact the composition of miRNAs; the level of several miRNAs, including miR16, is changed after 24 to 72 h storage either in the situation of 4°C or −20°C (McDonald et al., 2011) and brings another challenge in sample processing. That is to say, different experimental setups and processes all lead to the bias in the final output of miRNAs (Cortez et al., 2011; Schwarzenbach et al., 2014; Lee et al., 2016). Validated and optimized experimental protocols are needed urgently.

Second, the source of samples is also one of the most critical aspects of the ultimate results of circulating miRNAs (Wang et al., 2012). The expression of miRNAs is different between the samples extracted from the serum and plasma even in the same individual. The total RNA concentration is higher in the serum than in the plasma, which may be due to the RNA released from blood cells and platelets (Wang et al., 2012). However, analysis results from studies did not distinguish sample types (serum/plasma) when grouping.

Third, it is still hard to measure circulating miRNAs accurately because of its low concentration and existing form. The major quantifiability detection of circulating miRNAs are qPCR, microarray, and next-generation sequencing (NGS). Quantitative PCR is limited by low throughputs. It is now widely used for the verification of sequencing data. Microarray is influenced by the short length and similar sequence among clusters and families of mature miRNAs. Its requirement of pre-amplification step has a risk of alliterating the actual concentration of circulating miRNAs (Chen et al., 2009). NSG can meet the low concentration of circulating miRNAs due to its low input request and become a preferred method because of its lower cost and higher throughputs. Therefore, it is indispensable to unify the measurement methods and eliminate the deviation.

Additional obstacle lies in the normalization of data, especially the selection of internal control. U6 is widely used for intracellular miRNAs, but it is restricted due to its low expression in body fluids (Singh et al., 2016). Mir-16 was mentioned in many studies, and the inconsistent results also appear in multiple myeloma (Wang and Chen, 2014). Other internal controls, such as UNR6B (Xiang et al., 2014) and miR39 (Wulfken et al., 2011), are not trustworthy yet. The additive artificial non-human miRNAs external control like cel-miR-39 and cel-miR-54 (Zhong et al., 2018) could be a choice to solve this problem. However, it is hard to balance the amount among different samples. On the other hand, because of the expression of extracellular miRNAs in healthy individuals and the variability in acute/chronic inflammation/injury, the identification of a range of negative and cancer-specific "diagnostic" miRNAs is necessary before miRNAs can become clinical test indicators (Zhao et al., 2010; Xiang et al., 2014).

Cognitive Challenges

In addition to the technical challenges, the unclear understanding of the function and biology characteristics of circulating miRNAs, such as the secretion and transportation mechanisms, the cell to cell communication, the complicated network between miRNAs and coding gene, and the effects in upstream/downstream pathways are great barriers before clinical transformation (Wang and Chen, 2014). In the past few years, many researchers have devoted to the precise mechanisms of the secretion and uptake of miRNAs. However, it is still not clear if the package of miRNAs is random or specific; the release of cell-free miRNAs is passive or active. Besides, molecules and signals that are involved in the regulation of miRNAs, the precise role of circulating miRNAs in oncogenesis, the great heterogeneity of miRNAs in each type of cancer, different tumor stages, treatment response, and survival are all tasks that require more investigations.

At the same time, there are still challenges in the usage of circulating miRNAs in targeted therapy. Packaged artificial and modified miRNAs in exosomes could increase the stability of miRNAs *in vivo* (Cortez et al., 2011). However, the restricted tissue specificity and permeability is a big problem. Ligand, antibody, and nanoparticles that carried miRNAs are designed nowadays with improved specificity and decreased immunotoxicity (Chen et al., 2015). Nevertheless, a large number of preclinical studies in animals should be considered to verify their effectiveness.

CONCLUSIONS AND PERSPECTIVES

Since the first discovery of circulating miRNAs, there is a large amount of studies focused on their biological functions and the potential of biomarkers in oncology. As described here, miRNAs in the circulation change as molecular labels of tumor cells throughout the tumorigenesis and development of cancer. Such detectable changes make circulating miRNAs promising non-invasive biomarkers for early cancer diagnosis and predictor of prognosis and cancer treatment. However, several issues including technical and non-technical constraint need to be solved urgently. The further understanding of existing form in circulation and biological function, the deeper exploration of the underlying mechanism of release, transport, and uptake, and the special status in cell communication are all essential before the breakthrough in the application of circulating miRNA-based cancer therapy.

AUTHOR CONTRIBUTIONS

RC and XZ conceived the idea. MC wrote the manuscript. HW and XY revised the manuscript. DZ and YX edited the manuscript.

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Development and Validation of a 9-Gene Prognostic Signature in Patients With Multiple Myeloma

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Background: Multiple myeloma (MM) is one of the most common types of hematological malignance, and the prognosis of MM patients remains poor.

Objective: To identify and validate a genetic prognostic signature in patients with MM.

Methods: Co-expression network was constructed to identify hub genes related with International Staging System (ISS) stage of MM. Functional analysis of hub genes was conducted. Univariate Cox proportional hazard regression analysis was conducted to identify genes correlated with the overall survival (OS) of MM patients. Least absolute shrinkage and selection operator (LASSO) penalized Cox proportional hazards regression model was used to minimize overfitting and construct a prognostic signature. The prognostic value of the signature was validated in the test set and an independent validation cohort.

Results: A total of 758 hub genes correlated with ISS stage of MM patients were identified, and these hub genes were mainly enriched in several GO terms and KEGG pathways involved in cell proliferation and immune response. Nine hub genes (HLA-DPB1, TOP2A, FABP5, CYP1B1, IGHM, FANCI, LYZ, HMGN5, and BEND6) with non-zero coefficients in the LASSO Cox regression model were used to build a 9-gene prognostic signature. Relapsed MM and ISS stage III MM was associated with high risk score calculated based on the signature. Patients in the 9-gene signature low risk group was significantly associated with better clinical outcome than those in the 9-gene signature high risk group in the training set, test, and validation set.

Conclusions: We developed a 9-gene prognostic signature that might be an independent prognostic factor in patients with MM.

Keywords: multiple myeloma, weighted co-expression network analysis, prognostic signature, LASSO Cox proportional hazards regression model, survival

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INTRODUCTION

Multiple myeloma (MM), originated from malignant plasma cells secreting monoclonal M protein, represents the second most common malignancy in hematological malignancies (1–3). MM is differentiated from monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) by the presence of end-organ damage (4, 5). The clinical

symptoms of MM range from asymptomatic forms to manifestations of anemia, bone pain, and eventually spontaneous of fractures, renal failure, and frequent infections (6–8). Thanks to the introduction of novel agents (proteasome inhibitors, immunomodulatory drugs, and monoclonal antibodies), the management strategies for MM have been improved considerably in the past decade (9–12). Accordingly, the clinical outcomes of patients with MM have been significantly improved, however, MM remains an incurable disease and the prognosis of patients with MM remains poor (with a median survival of approximately 3–4 years) (13).

The International Staging System (ISS) stage of MM, based on β_2-microglobulin (β_2M) and albumin (ALB), divides MM patients into three different stages with significant dissimilar clinical outcomes. The ISS was a collaborative effort by investigators from 17 institutions worldwide and from data on 11,171 patients (4, 14). Patients with stage 1, 2, 3 diseases have median survivals of 62, 44, 29 months, respectively (4, 15).

Weighted gene co-expression network analysis (WGCNA), a systems biology algorithm that can be applied to describing the correlation patterns among genes across microarray samples, finding and summarizing modules of high related genes, and relating modules to certain clinical phenotype (16, 17), is widely used to facilitate the screening or identification of candidate biomarkers or therapeutic targets (18). Therefore, in the present study, we used WGCNA to screen potentially relevant molecular biomarkers correlated with the ISS stages of patients with MM. Moreover, we developed and validated the associated signature in patients with MM.

METHODS

MM Microarray Data

MM gene expression data and clinical information were downloaded from gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Affymetrix gene expression profiles were performed using Affymetrix Human Genome U133 Plus 2.0 (HG-U133 Plus_2.0) [GSE19784 (19) and GSE24080 (20)], Affymetrix Human Genome U133A Array(GSE6477) (21, 22), and Affymetrix GeneChip Human Gene 1.0 ST Array[E-MTAB-4032 (23)]. GSE19784, including 328 samples from patients with newly diagnosed MM, was used to construct the co-expression network. GSE24080, including 559 untreated MM samples, was randomly assigned patients in a 3:2 ratio to a training set and test set to develop and validate a prognostic signature. GSE6477, including 15 samples of normal donors, 73 samples of newly diagnosed MM, and 28 samples of relapsed MM, was used to evaluate the risk score calculated based on the prognostic signature among normal donor, newly diagnosed MM, and relapsed MM. E-MTAB-4032 (including 151 untreated MM) was used as an independent validation cohort to evaluate the prognostic role of the signature. Raw data of GSE19784 and E-MTAB-4032 were preprocessed using the R/Bioconductor "affy" package (24) and oligo (25) package, respectively. Robust Multi-array Average (RMA) (26) normalized data of these two studies at gene level were analyzed. For GSE6477 and GSE24080, the raw data had been normalized using MAS5 method and the expression levels of genes were transformed using the logarithm function. Purified CD138+ plasma cells (including myeloma cells and normal plasma cells) in GSE19784, GSE6477, E-MTAB-4032, and GSE20480 were separated using positive magnetic cell sorting selection with CD138 magnetic microbeads and subjected to gene expression profiling (GEP) as mentioned previously (19–23).

Construction of Co-expression Network and Identification of Hub Genes

The R package "WGCNA" (27) was used to construct a co-expression network for genes with highest variances (top 10000) in GSE19784. Prior to constructing the co-expression network, we applied sample networks method which was introduced by Oldham et al to detect outliers (28). A sample was considered as outlying, if the associated Z.K value was <-2.5. The soft threshold power β was selected according to the scale-free topology criterion as introduced previously (16, 17). Subsequently, Pearson's correlations between each gene pair was calculated to generate a matrix of adjacencies, and then the adjacencies were transformed into topological overlap matrix (TOM) (29). Next, we conducted average linkage hierarchical clustering based on the TOM-based dissimilarity. The minimum module size and medium sensitivity was 30 and 2, respectively, and other parameters were default. After relating modules to the ISS stage of MM patients and calculating the associated Gene Significance (the correlation between the genes and the trait) and Module Membership (the correlation of the first principal component of the expression matrix of the corresponding module and the gene expression profile), we screened hub genes using a networkScreening function based on Gene Significance and Module Membership and genes with q.Weighetd value (q-value (local FDR) calculated from weighted *p*-value of association with the ISS stage of MM) <0.01 were finally treated as hub genes (30).

Functional Enrichment Analysis of Hub Genes

To understand the biological function of the hub genes, we performed gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using the DAVID (31) online tool. GO and KEGG terms at $P < 0.05$ and false discovery rate (FDR)<0.05 were considered significantly enriched and the significantly enriched GO and KEGG terms were visualized using R package "ggplot2" (32).

Development of the Prognostic Signature Based on the Hub Genes

To investigate the associations between the hub genes and the survival of MM patients, we performed univariate Cox proportional hazards regression model in GSE24080. Genes significantly correlating with the overall survival (OS) of MM patients were included in a Least absolute shrinkage and selection operator (LASSO) penalized Cox proportional hazards regression model to minimize overfitting, and a 10-fold cross

validation was also conducted using the R package glmnet (33, 34). Then, we calculated the risk score for each patient based on this penalized Cox proportion model in the training set.

Validation of the Predictive Value of the Prognostic Signature in MM Patients

To validate the predictive value of the prognostic signature, Kaplan-Meier survival analysis, and univariate and multivariable Cox proportional hazards regression model were performed in the training set and test set in terms of OS, and event-free survival (EFS). Prior to multivariable Cox proportional hazards regression analysis on the OS, and EFS, we performed a variable selection based on the LASSO penalized Cox proportional hazards regression model. The definitions of OS and EFS was introduced previously (21–23). Meanwhile, we also validated the performance of the signature in the independent cohort E-MTAB-4032. The above survival analyses were conducted using the R packages "survival" (35) and "survminer" (version 0.4.3). MM patients in GSE24080 and E-MTAB-4032 were classified into the prognostic low risk group and the 9-signature high risk group based on the cutoff calculated through time dependent receiver operating characteristic (ROC) analysis using the R package "survivalROC" (36). The risk score of the signature in patients with ISS I, II, and III disease were evaluated using E-MTAB-4032. Meanwhile, the risk score of the signature in normal plasma cells, untreated MM, and relapsed MM were evaluated using GSE6477. The risk scores of the signature in E-MTAB-4032 and GSE6477 were presented as mean ± the standard error of the mean (SEM). Grouped data was analyzed using unpaired T-test, and $P < 0.05$ was considered statistically significant.

RESULTS

Results of Data Preprocessing, Co-expression Network Construction and Hub Genes Identification

No sample was demonstrated to be an outlier after all samples were clustered based on their Euclidean distances. Meanwhile,

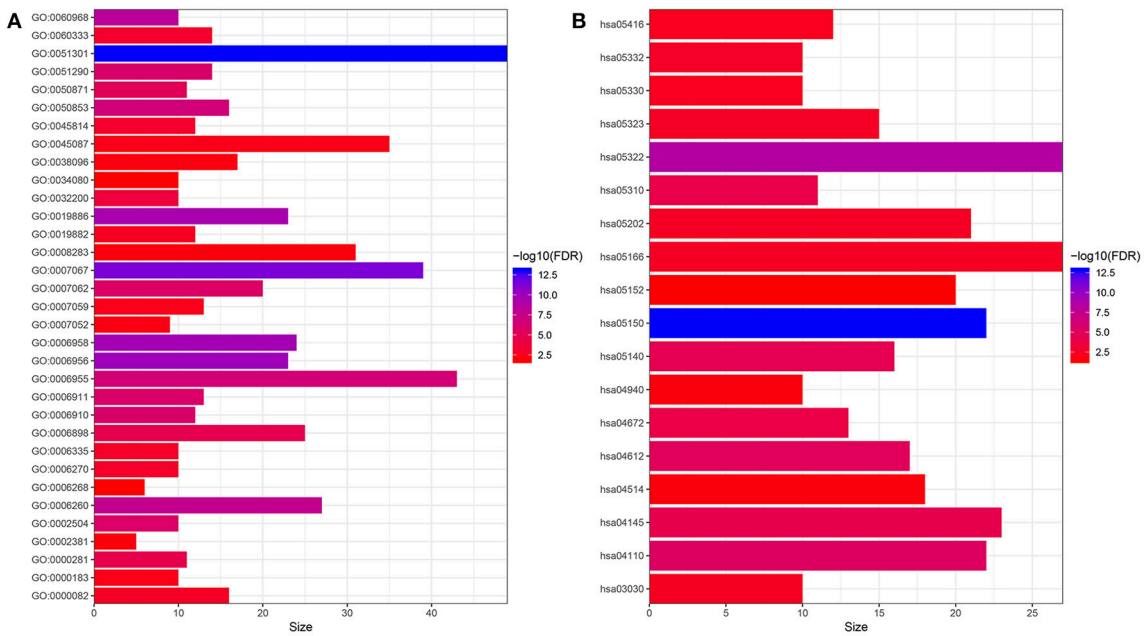

FIGURE 1 | Functional enrichment analysis of hub genes. **(A)** GO enrichment analysis. **(B)** KEGG enrichment analysis. GO:0051301~cell division, GO:0007067~mitotic nuclear division, GO:0006956~complement activation, GO:0006958~complement activation, classical pathway, GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II, GO:0060968~regulation of gene silencing, GO:0006260~DNA replication, GO:0050853~B cell receptor signaling pathway, GO:0006955~immune response, GO:0051290~protein heterotetramerization, GO:0006910~phagocytosis, recognition, GO:0006911~phagocytosis, engulfment, GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II, GO:0007062~sister chromatid cohesion, GO:0050871~positive regulation of B cell activation, GO:0006898~receptor-mediated endocytosis, GO:0000281~mitotic cytokinesis, GO:0032200~telomere organization, GO:0060333~interferon-gamma-mediated signaling pathway, GO:0045814~negative regulation of gene expression, epigenetic, GO:0006335~DNA replication-dependent nucleosome assembly, GO:0006270~DNA replication initiation, GO:0000082~G1/S transition of mitotic cell cycle, GO:0019882~antigen processing and presentation, GO:0007059~chromosome segregation, GO:0000183~chromatin silencing at rDNA, GO:0045087~innate immune response, GO:0007052~mitotic spindle organization, GO:0038096~Fc-gamma receptor signaling pathway involved in phagocytosis, GO:0008283~cell proliferation, GO:0002381~immunoglobulin production involved in immunoglobulin mediated immune response, GO:0006268~DNA unwinding involved in DNA replication, GO:0034080~CENP-A containing nucleosome assembly; hsa05150:Staphylococcus aureus infection, hsa05322:Systemic lupus erythematosus, hsa04110:Cell cycle, hsa04612:Antigen processing and presentation, hsa05140:Leishmaniasis, hsa04145:Phagosome, hsa05310:Asthma, hsa04672:Intestinal immune network for IgA production, hsa05332:Graft-vs.-host disease, hsa05166:HTLV-I infection, hsa05202:Transcriptional misregulation in cancer, hsa05323:Rheumatoid arthritis, hsa03030:DNA replication, hsa05330:Allograft rejection, hsa05416:Viral myocarditis, hsa04940:Type I diabetes mellitus, hsa04514:Cell adhesion molecules (CAMs), hsa05152:Tuberculosis.

$\beta = 12$, the lowest power for which the scale-free topology fit index reaches 0.9, was used for the subsequent adjacency calculation. After TOM based clustering, 14 gene modules were obtained. After co-expression network construction, a total of 780 probes were identified based on our screening criteria, 758 of which annotated to gene symbol were treated as hub genes (**Supplementary Table 1**). The major process of co-expression network construction and hub gene identification was shown in **Supplementary Figure 1**.

GO and KEGG Pathway Enrichment Analysis of Hub Genes

In order to have a preliminary understanding of the biological significance, we conducted GO and KEGG enrichment analysis. As shown in **Figure 1**, the hub genes were mostly enriched in GO terms related to cell proliferation ("cell division," "cell proliferation," "mitotic nuclear division," "DNA replication," "DNA unwinding involved in DNA replication," "sister chromatid cohesion," "mitotic cytokinesis," "DNA replication-dependent nucleosome assembly," "DNA replication initiation," "G1/S transition of mitotic cell cycle," "chromosome segregation," and "mitotic spindle organization") and immune response ("complement activation," "antigen processing and presentation of exogenous peptide antigen via MHC class II," " B cell receptor signaling pathway," " immune response," "phagocytosis, recognition," " positive regulation of B cell activation," "receptor-mediated endocytosis," "interferon-gamma-mediated signaling pathway," "innate immune response," "Fc-gamma receptor signaling pathway involved in phagocytosis,"and "immunoglobulin production involved in immunoglobulin mediated immune response") (**Figure 1A**). Furthermore, the results of KEGG pathway enrichment analysis of the hub genes suggested that these genes were mainly enriched in infection or immune related pathways ("Staphylococcus aureus infection," "antigen processing and presentation," "leishmaniasis," "asthma," "intestinal immune network for IgA production," "graft-vs.-host disease," "HTLV-I infection,"and "Rheumatoid arthritis"), and cell proliferation ("cell cycle,"and "DNA replication") (**Figure 1B**).

Development of a 9-Gene Signature in Patients With MM

To investigate the prognostic value of the hub genes, we conducted univariated Cox proportional hazards regression analysis, and the results suggested that the expression of 325 hub genes were significantly correlated with the OS of MM patients in the training set of GSE24080. To avoid overfitting as much as possible, we conducted LASSO penalized Cox proportional hazards regression model in the training set in GSE24080, and the results identified 9 of the 325 hub genes (HLA-DPB1(major histocompatibility complex, class II, DP beta 1), TOP2A (topoisomerase 2A), FABP5 (Fatty Acid-Binding Protein 5), CYP1B1(cytochrome P450 family 1 subfamily B member 1), IGHM (immunoglobulin heavy constant mu), FANCI (FA complementation group I), LYZ (lysozyme), HMGN5 (high mobility group protein N5 subtype), and BEND6 (BEN domain containing 6) with non-zero coefficient. Thus, we built the 9-gene signature on the basis of the coefficients of these 9 hub genes in the LASSO penalized model (**Supplementary Table 2**).

Associations Between the 9-Gene Signature and the ISS Stage of MM and the Disease Status

Firstly, we calculated the risk score of each MM patients for the 9-gene signature in GSE6477 and E-MTAB-4032 based on the coefficients of the 9 hub genes (**Supplementary Table 2**). As shown in **Figure 2A**, the risk score of patients with ISS stage III disease was significantly higher than that of patients with ISS stageI ($t = -0.362$, $P = 0.001$) and II ($t = -0.218$, $P = 0.031$). Meanwhile, the risk score of patients with relapsed MM was significantly higher compared with that of normal donor ($t = 5.782$, $P < 0.001$) and patients with untreated MM ($t = 2.977$, $P = 0.004$), and the risk score were higher in patients with untreated MM compared with that in normal donor ($t = -4.13$, $P < 0.001$, **Figure 2B**).

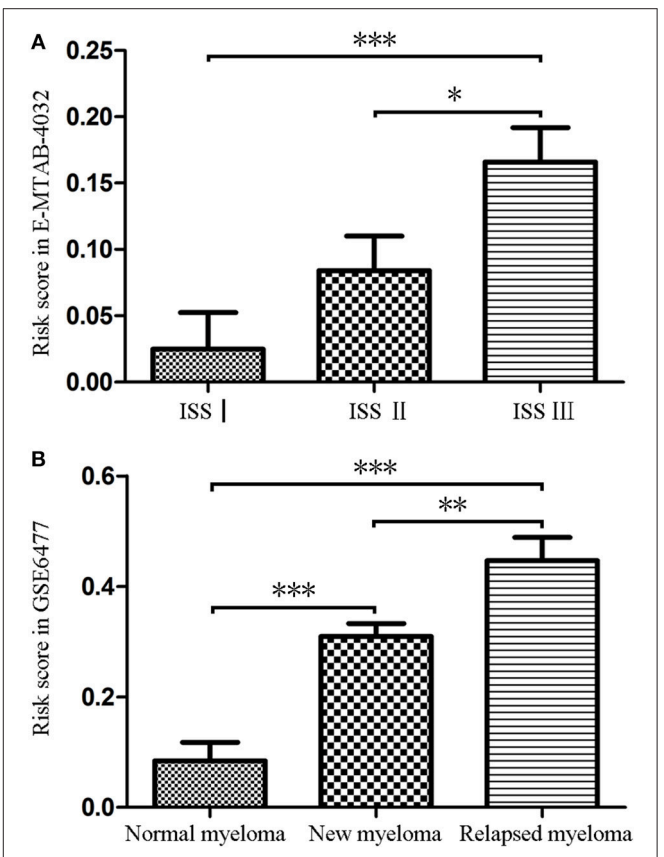

FIGURE 2 | The risk score of the signature in GSE6477 and E-MTAB-4032. **(A)** The risk score was increased in MM patients with ISS III disease and ISS IIcompared with that in MM patients with ISS Idisease. **(B)** The risk score of was significantly upregulated in newly diagnosed MM and relapsed MM compared with that in normal plasma cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Evaluation the Prognostic Role of the 9-Gene Signature

The predictive value of the 9-gene signature was evaluated in the training set, test set and an independent set E-MTAB-4032. Based on the optimal cutoff (1.939) calculated using time dependent ROC analysis (**Supplementary Figure 1**). The Kaplan-Meier survival analysis suggested that patients in the 9-gene signature low risk group had better OS compared with those in the 9-gene signature high risk group in the training set (HR = 0.2664, 95% CI: 0.1772-0.4007, log-rank $P < 0.001$, **Figure 3A**, **Supplementary Table 3**) and the test set (HR = 0.5115, 95% CI: 0.3137–0.8339, log-rank $P = 0.0062$, **Figure 3B**, **Supplementary Table 4).** Meanwhile, patients in the 9-gene signature low risk group had with better EFS compared with those in the 9-gene signature high risk group in the training set (HR = 0.3321, 95% CI:0.2395–0.4606, $P < 0.0001$, **Figure 3C**, **Supplementary Table 5**) and test set (HR = 0.5174, 95% CI: 0.3447–0.7765, $P = 0.0015$, **Figure 3D**, **Supplementary Table 6**). Based on the results of variable selection (**Supplementary Table 7**), age, B2M (β2-microglobin), CRP (C reaction protein), LDH (lactate dehydrogenase), BMPC (bone marrow plasma cell), MRI (magnetic resonance imaging), and the 9-gene signature was included multivariable Cox proportional hazards regression analysis which indicated that the 9-gene signature was an independent prognostic factor in terms of OS and EFS in the training set and test (**Supplementary Tables 3–6**). Moreover, MM patients in the 9-gene low risk group also had better OS compared with those in the 9-gene signature high risk group in the independent validation cohort E-MTAB-4032 (HR = 10.6091, 95% CI: 3.2120–35.0409, log-rank $P = 0.0061$, **Figure 4**). Meanwhile, the results of multivariable

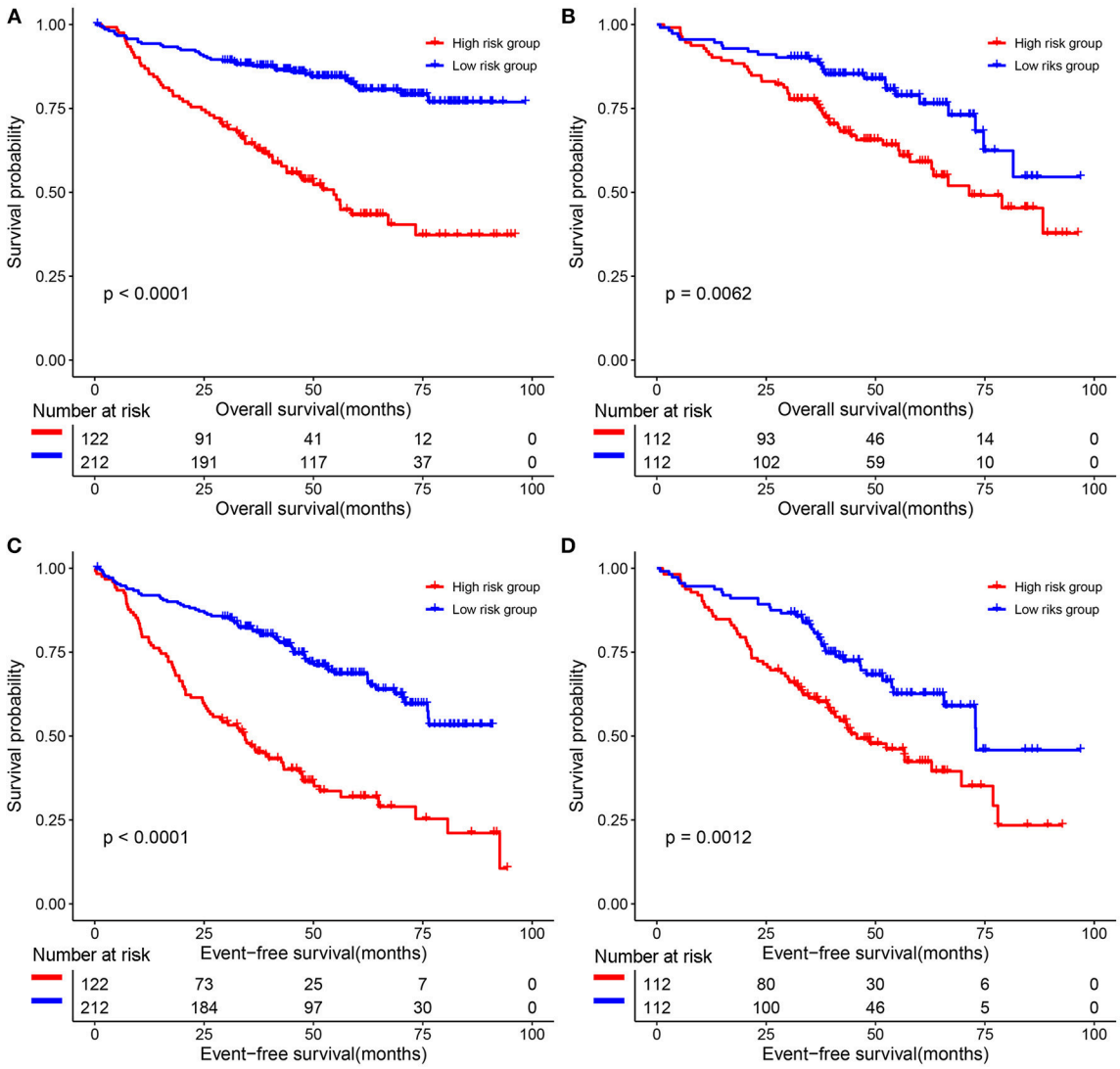

FIGURE 3 | The correlations between the 9-gene signature and the overall survival (OS) and event-free survival (EFS) of patients with MM. **(A)** OS in the training set. **(B)** OS in the test set. **(C)** EFS in the training set. **(D)** EFS in the test set.

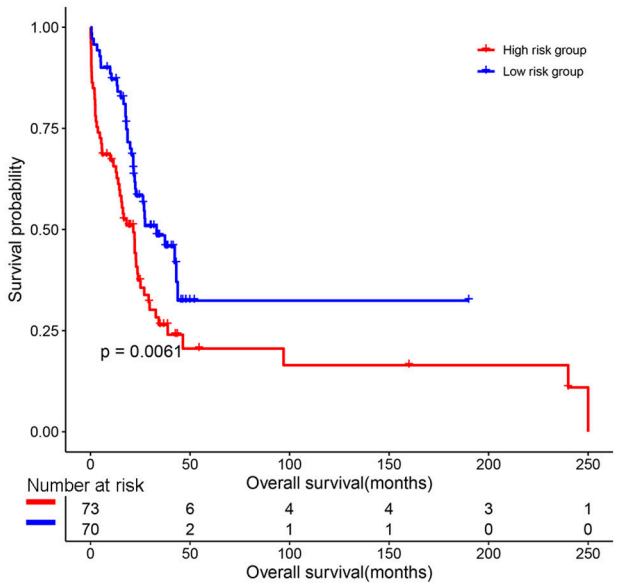

FIGURE 4 | The prognostic role of the 9-gene signature in the independent validation cohort E-MTAB-4032.

Cox proportional hazards suggested that the 9-gene signature was also an independent prognostic factor in the validation cohort (HR = 14.8092, 95% CI:1.2282–178.5591, $P = 0.0339$, **Supplementary Table 7**).

DISCUSSION

As stated above, although several novel agents for patients with MM have been introduced into clinical practice, the disease remained incurable, and the clinical outcome of patients with MM is still poor (2, 3). Thus, it is of vital importance to develop such molecular biomarkers or signature that are significantly correlated with the clinicopathological features and clinical outcome of patients with MM. In the present study, a total of 758 hub genes associated with the ISS stage of MM patients were identified through WGCNA. Thus, we performed univariate Cox proportional hazards regression analysis to analyzed the relations between the expression of hub genes and the OS of patients with MM, and the results suggested that 325 hub genes were associated with the OS of MM patients. LASSO (34) was introduced in order to improve the prediction accuracy and interpretability of regression models by forcing the sum of the absolute value of the regression coefficients to be less than a fixed value, which forces certain coefficients to be set to zero, effectively choosing a simpler model that does not include those coefficients. Based on this, we included the above 325 hub genes into a LASSO penalized Cox proportional hazards regression model, as a result, 9 hub genes with none-zero coefficients in this model was identified. Thus, we calculated the risk score of each MM patients, and built the 9 hub genes [HLA-DPB1(major histocompatibility complex, class II, DP beta 1), TOP2A (topoisomerase 2A), FABP5 (Fatty Acid-Binding Protein 5), CYP1B1(cytochrome P450 family 1 subfamily B member 1), IGHM (immunoglobulin heavy constant mu), FANCI (FA complementation group I), LYZ (lysozyme), HMGN5 (high mobility group protein N5 subtype), and BEND6 (BEN domain containing 6)] based signature.

Results of functional enrichment analysis of hub genes suggested that hub genes were mainly enriched in cell proliferation and immune response related GO terms and pathways, this was in accordance with the prognostic value of the 9-gene signature developed based on the hub genes. MM patients in the 9-gene low risk group was associated with better OS and EFS compared with those in the 9-gene high risk group, and the 9-gene signature was shown to be an independent prognostic signature in patients with MM.

Actually, most of these hub genes in the signature had been demonstrated be associated with the proliferation or invasion of several human cancers. HLA-DPB1, also known as major histocompatibility complex, class II, DP beta 1, belongs to the HLA-class IIbeta chain paralogue. Li et al. demonstrated that genetic variant of HLA-DPB1 increased in the risk of extranodal natural killer T-cell lymphoma (37). Liu et al. demonstrated that TOP2A (topoisomerase 2A) and TOP1 functioned as oncogene in liver cancer (38). Meanwhile, decreased expression of TOP2A inhibited the proliferation of invasion of colon cancer cells (39). Kawaguchi et al and Powell et al. demonstrated that overall expression of FABP5 promoted the proliferation and metastasis of colorectal cancer cells and breast cancer cells (40). Gu et al. demonstrated that genetic variant of CYP1B1 gene was associated prognosis of patients with prostate cancer (41). Mutation patterns of IGHM was associated different progression pathways in follicular lymphoma (42). Chen et al demonstrated that PRC1 could promote early recurrence of patients with hepatocellular carcinoma by regulating the expression of FANCI (43). Mariano FV et al demonstrated that the expression of LYZ could be used to differentiate mammary analog secretory carcinoma from acinic cell carcinoma of salivary glands (44). Wu et al demonstrated that the expression of HMGN5 was increased in bladder cancer cells and high expression of HMGN5 was associated with poor prognosis of patients with bladder cancer (45). The results of the above literature review provided strong support for the 9-gene signature in the clinical out prediction in patients with MM.

Limits of our study are as follows. First, our study is a retrospective analysis based on previously published MM gene expression studies, although its conclusions have been confirmed in the test set and independent validation set, we recommend that the conclusions of this study should be verified by molecular biology experiments in subsequent studies. Second, the prognostic performance of the 9-gene signature should be evaluated through prospective clinical trials.

Taken together, we developed a 9-gene prognostic signature based on the hub genes obtained through a co-expression

network, patients in the 9-gene signature low risk group were associated with better clinical outcomes compared with those in the 9-gene signature high risk group.

AUTHOR CONTRIBUTIONS

X-PL collected and analyzed the data, write the manuscript. Xia-HY analyzed the data, and review the manuscript. X-YM and Xin-HY participated in revising the manuscript. FW revised the manuscript, analyzed the data, and participated in preparation of the figures. LH designed the study and participated in data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00615/full#supplementary-material

Supplementary Figure 1 | Flowchart depicting the major process of Co-expression network construction and identification of hub genes.

Supplementary Figure 2 | Time-Dependent ROC Curve for the 9-gene signature in the training set at 90 months.

Supplementary Table 1 | Hub genes calculated using weighted co- expression network analysis and their impact on the overall survival of patients with multiple myeloma.

Supplementary Table 2 | The 9 hub genes with non-zero coefficients in the LASSO Cox proportional hazards regression model.

Supplementary Table 3 | Cox proportional hazards regression analysis the association between the clinical characteristics and overall survival of multiple myeloma in the training set.

Supplementary Table 4 | Cox proportional hazards regression analysis on the association between the clinical characteristics and overall survival of multiple myeloma patients in the test. set.

Supplementary Table 5 | Cox proportional hazards regression analysis on the association between the clinical characteristics and event-free survival of multiple myeloma patients in the training set.

Supplementary Table 6 | Cox proportional hazards regression analysis on the association between the clinical characteristics and event-free survival of multiple myeloma patients in the test set.

Supplementary Table 7 | The prognostic role of the 9-gene signature in the independent validation cohort.

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Detection of Exosomal PD-L1 RNA in Saliva of Patients With Periodontitis

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Periodontitis is the most prevalent inflammatory disease of the periodontium, and is related to oral and systemic health. Exosomes are emerging as non-invasive biomarker for liquid biopsy. We here evaluated the levels of programmed death-ligand 1 (*PD-L1*) mRNA in salivary exosomes from patients with periodontitis and non-periodontitis controls. The purposes of this study were to establish a procedure for isolation and detection of mRNA in exosomes from saliva of periodontitis patients, to characterize the level of salivary exosomal *PD-L1*, and to illustrate its clinical relevance. Bioinformatics analysis suggested that periodontitis was associated with an inflammation gene expression signature, that *PD-L1* expression positively correlated with inflammation in periodontitis based on gene set enrichment analysis (GSEA) and that *PD-L1* expression was remarkably elevated in periodontitis patients versus control subjects. Exosomal RNAs were successfully isolated from saliva of 61 patients and 30 controls and were subjected to qRT-PCR. Levels of *PD-L1* mRNA in salivary exosomes were higher in periodontitis patients than controls ($P < 0.01$). Salivary exosomal *PD-L1* mRNA showed significant difference between the stages of periodontitis. In summary, the protocols for isolating and detecting exosomal RNA from saliva of periodontitis patients were, for the first time, characterized. The current study suggests that assay of exosomes-based *PD-L1* mRNA in saliva has potential to distinguish periodontitis from the healthy, and the levels correlate with the severity/stage of periodontitis.

Keywords: immune checkpoint, exosomes, saliva, chronic periodontal disease, biomarker, disease stage

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INTRODUCTION

Periodontitis is one of the most prevalent disease in dentistry, impairing the integrity of the periodontium and leading ultimately to tooth loss. Periodontitis is a chronic inflammatory disease caused by microorganisms colonizing the dentogingival interface (Slots, 2013). Currently, the diagnosis of periodontitis was mostly based on clinical and radiographic evaluations without specific evaluation of the underlying inflammatory response (Buduneli and Kinane, 2011; Baeza et al., 2016). Measuring electrolyte concentration in gingival crevicular fluid (GCF), especially the

concentration of sodium, potassium and calcium ions, reflects the clinical status of the periodontal tissues such that the pattern of concentrations of these ions may be used as a potential diagnostic marker for active periodontitis (Koregol et al., 2011).

Given the close relevance of inflammation to periodontitis, immune-regulatory factors have been explored as biomarker of periodontitis (Han et al., 2012; Kimak et al., 2015). Programmed death-ligand 1 (PD-L1), also known as the B7-H1 receptor, plays an important role in cell-mediated immune responses (Gianchecchi et al., 2013). PD-L1 regulates T cell activation and tolerance, and is able to inhibit activated T cell functions and survival (Kim et al., 2005). High expression of PD-L1 in host cells may contribute to the chronicity of inflammatory disorders (Zamani et al., 2016).

PD-L1 is involved in periodontitis. *Porphyromonas gingivalis* (*P. gingivalis*) is a keystone pathogen in chronic periodontitis (Groeger et al., 2017), and it induces expression of PD-L1 in malignant and non-malignant oral epithelial cells (Groeger et al., 2017). In periodontitis, P. gingivalis inhibits the synthesis of cytokines and increases humoral responses. This reduces the inflammatory responses related to T- and B-cell activation, and subsequent IFN-γ secretion by a subset of T cells. The T cells that secrete IFN-γ further suppress upregulation of programmed cell death-1 (PD-1)-receptor and its ligand PD-L1 on CD11b+-subset of T cells (Gaddis et al., 2013; Groeger et al., 2017). Interestingly, studies recently have demonstrated elevated PD-L1 levels in saliva from patients with oral cancers or salivary gland carcinoma (Aziz et al., 2015; Goncalves et al., 2015, 2017). Saliva-derived samples have been studied as a source of biomarkers for periodontitis (Baltacioglu et al., 2014; Recker et al., 2015).

Exosomes are small (30—100 nm) membrane-encapsulated vesicles containing nuclei acids and protein cargo, and secreted by eukaryotic cells into the circulation (Tkach and Thery, 2016). The contents of disease cell-derived exosomes may potentially serve as a source of disease biomarkers. Salivary exosomal contents have recently been investigated for diagnosis and prognosis of a wide range of diseases (Michael et al., 2010; Lau et al., 2013; Byun et al., 2015; Machida et al., 2015; Sivadasan et al., 2015; Kim et al., 2017; Zheng et al., 2017; Han et al., 2018). Although detection of *PD-L1* mRNA has been reported in periodontitis (Yuan et al., 2015; Zhang et al., 2016), it is unknown whether *PD-L1* mRNA can be detected in exosomes of saliva of periodontitis patients and whether the level of salivary exosomal *PD-L1* mRNA reflects disease status. We therefore focused on exosomes and saliva, and set to establish a protocol for isolation and detection of exosomes, and exosomal RNA in the saliva of periodontitis patients, and to characterize salivary exosomal PD-L1 as a potential marker for periodontitis.

MATERIALS AND METHODS

Study Population

This study is a prospective observational investigation at the First Affiliated Hospital of Shantou University. From June, 2017 to June, 2018, 61 periodontitis patients and 30 control subjects were enrolled in the study with informed consent. Each recruited subject was inquired in detail about the medical history and accepted a thorough periodontal examination. Diagnosis of periodontitis was based on assessment of probing pocket depth (PD) and clinical attachment loss (CAL). Patients who fulfilled the requirement of generalized chronic periodontitis according to the classification by the American Academy of Periodontology in 2007 (Slots, 2013) were recruited to the periodontitis group after periodontal examination. The non-periodontitis control group was composed of subjects with no evidence of periodontitis after examination according to the above classification. Smoking and alcohol using status was recorded for all individuals. Exclusion criteria were age <18 years, inability to give informed consent, congenital malformation, chronic diseases (e.g., lip cancer, gingival cancer, carcinoma of the tongue, soft palate carcinoma, jaw bone cancers, cancers of the mouth floor carcinoma, oropharyngeal cancer, salivary gland carcinoma, maxillary sinus carcinoma, cancer occurs in facial ministry skin mucous membrane, epilepsy, cardiac disease, lung disease, renal disease, positive test for human immunodeficiency virus etc.), and history of systemic antibiotic treatment or dental prophylaxis in the previous 6 months. Written informed consents were obtained from all participants in accordance with the principles established by the Helsinki Declaration. This study was approved by the Institutional Review Board of Shantou University Medical College (SUMC) (Shantou, China) under IRB protocol number: #04-070.

Clinical Evaluation

For each individual, PD, CAL and bleeding on probing (BP) values were measured with a periodontal probe. A single calibrated examiner assessed these clinical parameters. Control individuals (control group) did not present any sign or symptom compatible with periodontal disease (PD $<$ 3 mm, CAL $<$ 3 mm and no radiographic evidence of alveolar bone breakdown). On the other hand, the periodontitis group consisted of patients with mild, moderate or severe periodontitis (based on the Classification of Periodontal Diseases and Conditions of Armitage, 2007) (Slots, 2013) with at least one single-rooted tooth with a CAL $\geq$ 6 mm and probing depth $\geq$ 5 mm.

Gingival Biopsies

Human gingivae were obtained from all periodontitis patients. Gingiva was harvested during tooth extractions for periodontal reasons in the Department of Stomatology of First Affiliated Hospital of Shantou University. Written informed consent and approval of the Ethics Committee of the SUMC were obtained.

Human Saliva Collection

Saliva was collected in the morning (8 am to 10 am) from all subjects. During the collection period, subjects were instructed to refrain from eating, drinking or using oral hygiene products for at least 1 h prior to collection, and received no stimulation of salivation. After rinsing the mouth with water, each subject spat 3–5 mL saliva into a 35-mm dish. Subjects were reminded not to cough up mucus, and saliva was collected within 30 min from spitting. These saliva samples were pipetted into 1.5-mL tubes and were kept on ice during processing which did not exceed

60 min. The samples were then centrifuged at 3,000 × *g* for 15 min at 4°C to remove cells and cellular fragments. As much supernatant as possible was collected into new 1.5-mL tubes and stored at −80°C.

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA; v2.09[1]) was performed to examine the association between gene sets and gene expression (Dong et al., 2017). Periodontitis gene expression profiles from an independent datasets (GSE16134) were collected from NCBI Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/. The expression levels of affected gingival tissue (n = 241) vs. unaffected gingival tissue (n = 69) from GSE16134 was ordered from high to low. We performed GSEA analysis to examine the correlation between periodontitis and the PD-L1 signature pathway, followed by the protocol available at the GSEA website[1].

Isolation of Salivary Exosomes

ExoQuick™ exosomes precipitation solution was used for exosomes isolation according to the manufacturer's instructions (System Biosciences, Mountain View, CA, United States). Briefly, ExoQuick-TC™ solution was added to saliva at a 63/250 ratio and mixed by inverting the tubes several times. Exosomes were precipitated by refrigeration at 4°C overnight, and then collected by centrifugation twice at 1,500 × *g* for 30 and 5 min, respectively, in order to remove the supernatant. Supernatant was discarded, and the pellet resuspended in 300 μL TRIZOL for RNA isolation or 20 μL PBS for protein isolation.

RT-qPCR

Total RNA was extracted from exosomes and cell lysates using TRIzol reagent as per the manufacturer's protocol (ZYMO RESEARCH). RNA was eluted with 25 μL of RNAse-free water. RT-qPCR was performed using an Absolute Blue QPCR SYBR Green Low ROX mix (Thermo Scientific) on an Applied Biosystems' 7500 real-time PCR system. The Rn value (normalized reporter value) was the fluorescent signal from SYBR Green normalized to the signal of the passive reference dye for a given reaction. No-template and no-RT reactions were performed as negative controls. All assays were performed in 3 separate RTs followed by triplicate qPCR, and the results are shown as the average fold change relative to GAPDH which served as an internal control. Primers for RT-qPCR were:

GAPDH F: 5′-TGCACCACCAACTGCTTAGC-3′
GAPDH R: 5′-GGCATGGACTGTGGTCATGAG-3′
PD-L1 F: 5′-TGCCGACTACAAGCGAATTACTG -3′
PD-L1 R: 5′-CTGCTTGTCCAGATGACTTCGG-3′.

Protein Isolation and Immunoblotting

Exosomal pellets were resuspended in PBS and re-pelleted by centrifugation and then extracted with RIPA buffer (Santa Cruz Biotechnology). Total protein lysates were prepared and analyzed by immunoblotting using anti-ALIX (Cat. No. 2171; Cell Signaling Technology, Beverly, MA, United States), anti-TSG101 (Cat. ab133586; Abcam, Cambridge, United Kingdom), anti-CD63 (Cat. sc-15363; Santa Cruz Biotechnology, CA, United States), anti-CD9 (Cat. sc-9148; Santa Cruz Biotechnology, CA, United States), anti-CD81 (Cat. ab109201; Abcam, Cambridge, United Kingdom), anti-Calnexin (Cat. No. 2433; Cell Signaling Technology, Beverly, MA, United States), anti-LC3 (Cat. No. 2775; Cell Signaling Technology, Beverly, MA, United States), anti-NLRP3 (Cat.19771-1-AP; Proteintech, United States) and anti-NLRP4 (Cat. NB100-56156; Novus Biologicals, United States) as described previously (Gan et al., 2016; Dong et al., 2017).

Transmission Electron Microscopy

Following exosomes isolation, the pellet was washed in PBS, and then subjected to ultracentrifugation at 120,000 × *g* for 70 min to re-pellet the exosomes. The exosomes pellets were resuspended in 30 μL PBS, and a 10 μL aliquot of the suspension loaded onto formvar carbon-coated grids and allow to stand for 5 min at room temperature. Next, the exosomes were fixed in 2% paraformaldehyde for 5 min at room temperature and washed thrice with PBS. Excess liquid was drained by gently touching the edge of the grid with clean filter paper. The grid was slightly touched onto a drop of 2% uranyl acetate for 1 min and embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%). Excess liquid was drained off, and then the grid was allowed to air dry for several minutes prior to examination under a transmission electron microscope (JEM-1400, Hitachi, Shiga, Japan) (Raposo and Stoorvogel, 2013).

Nanoparticle Tracking Analysis

Total particles in human salivary samples were analyzed by nanoparticle tracking by the A & P Instrument Co., Ltd. (Guangzhou, China), using a NanoSight LM10 system (NanoSight Ltd., Amesbury, United Kingdom). Each sample was diluted in nanoparticle-free PBS and analyzed three times. Data was collected and analyzed using the nanoparticle tracking analysis (NTA) software (RRID: SCR_014239, version 2.3). All measurements were recorded at room temperature.

Cell Culture

All cells were cultured in a sterile incubator maintained at 37°C with 5% CO2. ESCC cells and THP-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, United Kingdom) or RPMI-1640 medium (Gibco, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Paisley, United Kingdom), 10 mmol/L glutamine, 100 units/ml penicillin (Sigma, St. Louis, MO, United States), and 100 μg/ml streptomycin (Sigma). TE1 cells were provided by Dr. X.C. Xu (The University of Texas MD Anderson Cancer Center, Houston, TX, United States). For LC3 detection, TE1 cells were collected after starvation (i.e., without fetal bovine serum) for 6 h.

[1]http://www.broadinstitute.org/gsea/

Statistical Analysis

All statistical analyses were performed using the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, United States) and Prism V6.01 (GraphPad). Summary statistics reporting means and standard errors were stated as appropriate. Statistical methods used included *t*-test, Pearson correlation and chi-square test.

RESULTS

Bioinformatic Analysis of the Correlation Between Periodontitis and PD-L1

Given the association of periodontitis with inflammation, and the importance of PD-L1 in inflammatory immunity, we analyzed database to test whether periodontitis correlates with PD-L1. GSEA analysis revealed that periodontitis is positively associated with an inflammation signature, and PD-L1 is positively correlated with an inflammation signature in periodontitis (**Figure 1A**). Analysis of the GEO database (GSE16134) indicated a statistically significant elevation of PD-L1 level in periodontitis patients versus control subjects (**Figure 1B**). These data support the hypothesis that PD-L1 is positively relevant to periodontitis.

Patient Demographics and Clinical Parameters

This pilot study was conducted at Shantou University Medical College. Sixty-one periodontitis patients and 30 control subjects were enrolled in the study (**Figure 2**). With an average age of 51, 29 male and 32 female patients from the First Affiliated Hospital of Shantou University were recruited. There are also 30 control subjects (14 male and 16 female) were enrolled in this study with an average age of 52. Among demographic and clinicopathological characteristics, age, gender, tobacco use, alcohol use, hypertension and diabetes did not show any significant differences between the two groups (**Table 1**).

Extraction and Characterization of Exosomes From Human Saliva

Isolation of exosomes from human saliva was confirmed by transmission electron microscopy (TEM) (i.e., spherical membrane-bound particles with diameters between 30 and 100 nm) (Thery et al., 2009; Raposo and Stoorvogel, 2013; **Figure 3A**) and nanoparticle tracking analysis showing that human exosomes had an average diameter of 95 nm (Filipe et al., 2010; Webber and Clayton, 2013; **Figure 3B**). Immunoblotting of exosomal markers (ALIX, TSG101, CD63, CD9, CD81) and the intracellular protein that is not present in exosomes (Calnexin) (Mathivanan and Simpson, 2009; Taylor and Gercel-Taylor, 2011; Lotvall et al., 2014; **Figure 3C**) were performed for further confirmation. For eliminating the possibility of contamination by autophagosomes where significant amount of PD-L1 would be found, LC3 II, the marker of autophagosome was evaluated by immunoblotting; salivary exosomes samples did not contain LC3 while lysate of TE1 cells cultured in DMEM without serum for 6 h (positive control) showed the presence of LC3 II (**Figure 3C**). In addition, the marker of endoplasmic reticulum, calnexin, and the markers of inflammasomes were also analyzed by immunoblotting to exclude contamination of exosomes. The immunoblotting showed that calnexin, NLRP3

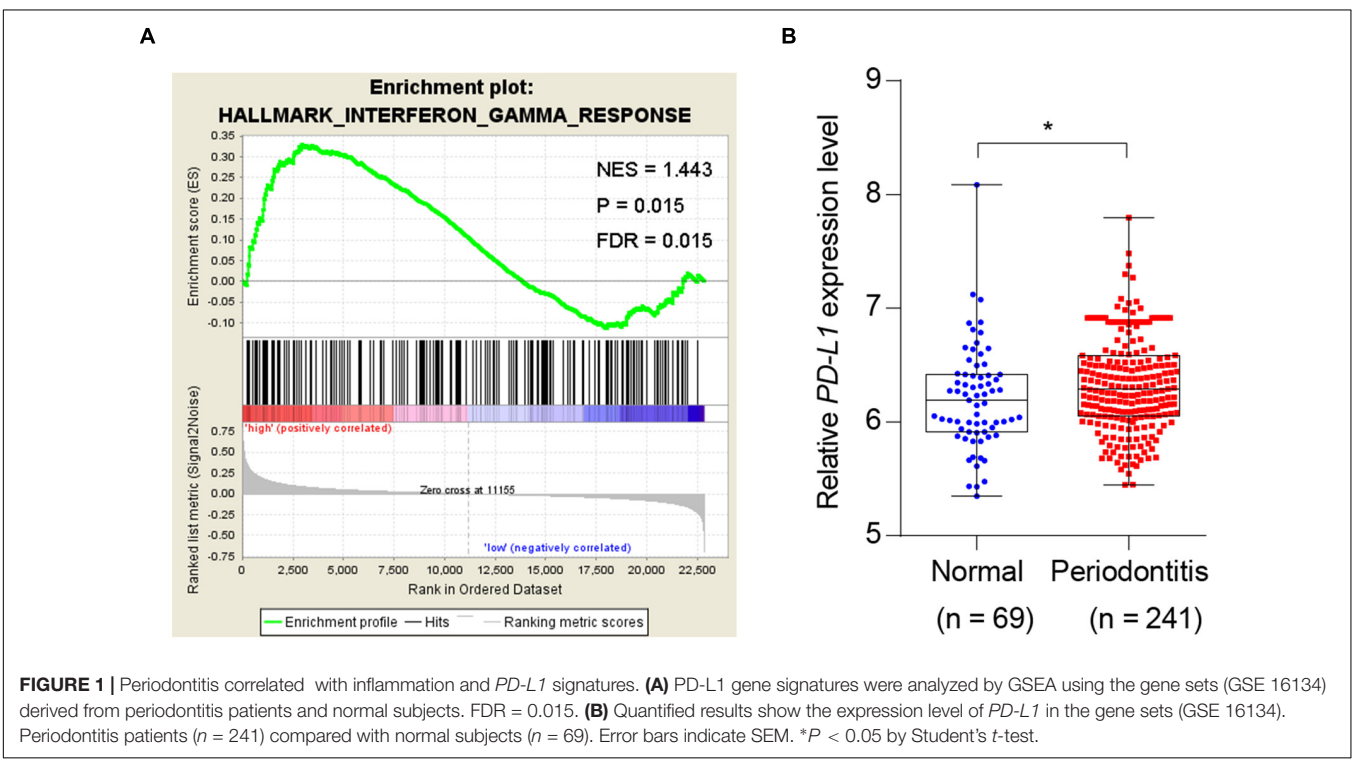

FIGURE 1 | Periodontitis correlated with inflammation and *PD-L1* signatures. **(A)** PD-L1 gene signatures were analyzed by GSEA using the gene sets (GSE 16134) derived from periodontitis patients and normal subjects. FDR = 0.015. **(B)** Quantified results show the expression level of *PD-L1* in the gene sets (GSE 16134). Periodontitis patients (n = 241) compared with normal subjects (n = 69). Error bars indicate SEM. *$P < 0.05$ by Student's *t*-test.

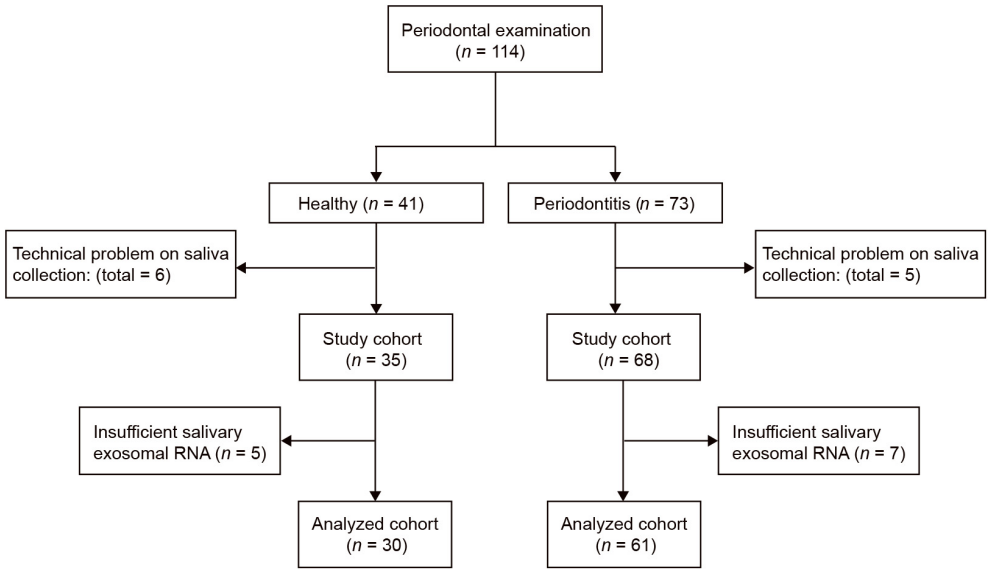

FIGURE 2 | Flow diagram of periodontitis patients and control subjects. Participant numbers are accounted for by the flow diagram for the study.

TABLE 1 | The demographics and clinicopathological characteristics of the periodontitis patients and control subjects.

Variables	Control (*n* = 30) *n* (%)	Patient (*n* = 61) *n* (%)	*P*-value*
Age (years)			
≤50	18 (31.6)	39 (68.4)	0.715
>50	12 (35.3)	22 (64.7)	
Gender			
Female	21 (39.6)	32 (60.4)	0.111
Male	9 (23.7)	29 (76.3)	
Tobacco use			
No	19 (32.2)	40 (67.8)	0.833
Yes	11 (34.4)	21 (65.6)	
Alcohol use			
No	20 (30.8)	45 (69.2)	0.481
Yes	10 (48.5)	16 (61.5)	
Hypertension			
No	23 (35.9)	41 (64.1)	0.353
Yes	7 (25.9)	20 (74.1)	
Diabetes			
No	27 (36.5)	47 (63.5)	0.136
Yes	3 (17.6)	14 (82.4)	
Stage			
Mild	NA	26	
Moderate	NA	21	
Severe	NA	14	

**P-values were calculated by χ^2 test.*

and NLRP4 could be detected in positive control but not in salivary exosomes samples (**Figure 3C**). Thus, exosomes were successfully isolated from saliva of periodontitis patients without significant contamination by autophagosomes, endoplasmic reticulum or inflammasomes.

Salivary Exosomal *PD-L1* mRNA Was Elevated in Periodontitis Patients

We next determined the salivary exosomal and gingival *PD-L1* mRNA expression, in 61 periodontitis patients and 30 control subjects, by qPCR analysis. As shown in **Figure 4A**, 45 of 61 cases (73.8%) had increased salivary exosomal *PD-L1* expression compared with the mean salivary exosomal *PD-L1* expression in control subjects. Mean salivary exosomal *PD-L1* expression in the periodontitis patients was found to be about 10-fold higher when compared with the paired control subjects ($P < 0.001$). Similarly, mean gingival *PD-L1* mRNA expression in the periodontitis patients was more than 10-fold higher than that in control subjects (**Figure 4B**, $P < 0.001$). More importantly, salivary exosomal *PD-L1* mRNA levels highly correlated with gingival *PD-L1* mRNA levels in periodontitis patients ($r = 0.800$ and $P < 0.001$, Pearson's correlation test; **Figure 4B**). Collectively, our results strongly suggested that salivary exosomal *PD-L1* mRNA could be a feasible biomarker of periodontitis.

Clinical Relevance of Salivary Exosomal *PD-L1* mRNA in Periodontitis Patients

We further assessed the association between salivary exosomal *PD-L1* mRNA and clinical parameters in 61 periodontitis patients. The median value (i.e., relative value of salivary exosomal *PD-L1* mRNA = 9.90) was chosen to classify patients into high-($n = 31$) and low-*PD-L1* ($n = 30$) groups (**Table 2**). High *PD-L1* expression was only associated with advanced stage ($P = 0.005$; χ^2 test; **Table 2**), and *PD-L1* expression was not found to be statistically significantly associated with other clinicopathological parameters (**Table 2**). Our results demonstrated that salivary exosomal *PD-L1* mRNA could reflect the stage of periodontitis, suggesting that PD-L1 may be relevant to the disease progression.

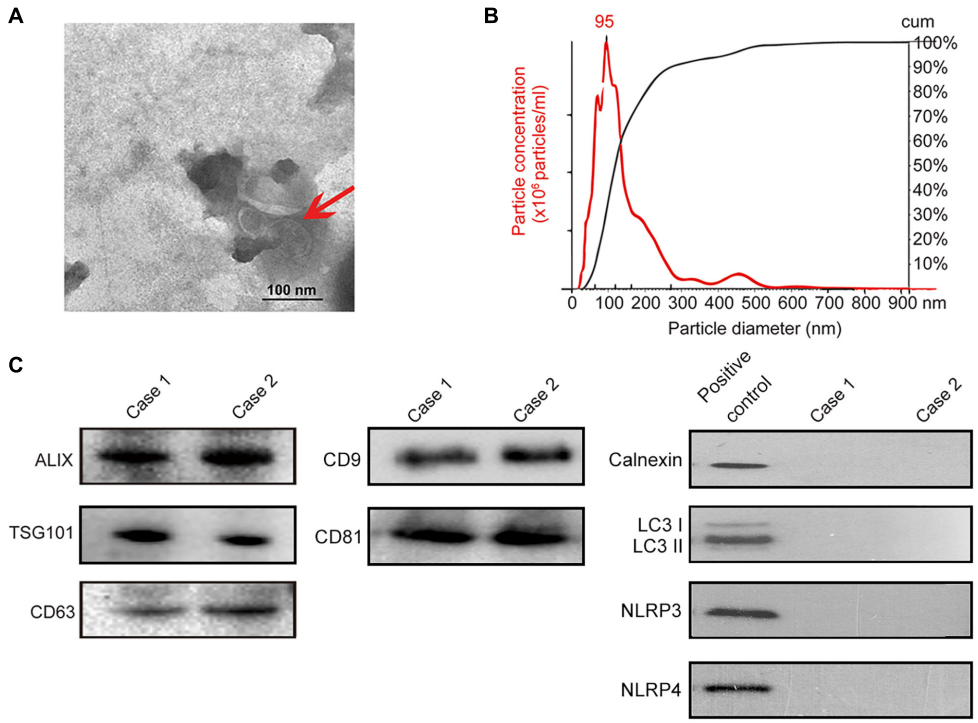

FIGURE 3 | Identification of salivary exosomes in periodontitis patients and normal subjects. **(A)** Transmission electron microscopy of exosomes isolated from human saliva. Scale bar: 100 nm. **(B)** Exosomes concentration and size distribution by NanoSight analysis of human saliva. **(C)** Immunoblotting showed the exosomal membrane markers (ALIX, TSG101 CD63, CD9 and CD81), the intracellular protein Calnexin, the marker of autophagosome LC3 and markers of inflammasome (NLRP3 and NLRP4) in exosomes isolated from the saliva of one normal subject (case 01) and one periodontitis patient (case 02). Positive control for Calnexin was TE1 cells, and positive control for LC3 was TE1 cells after starvation for 6 h. Positive control for NLRP3 and NLRP4 was THP-1 cells.

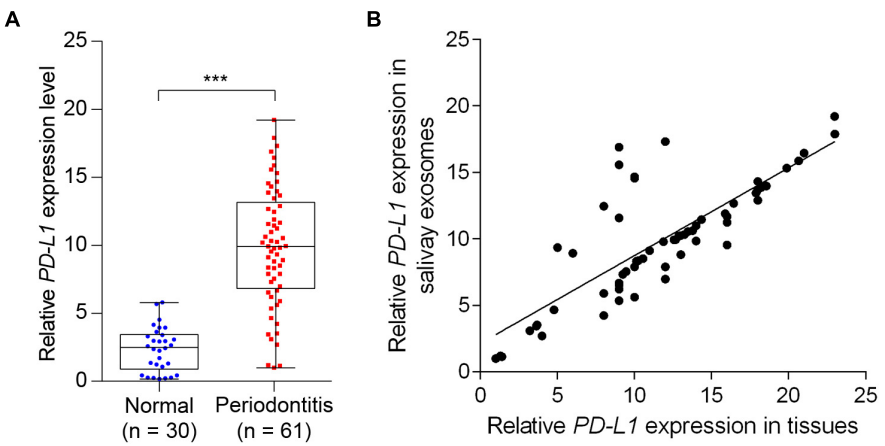

FIGURE 4 | Detection of salivary exosomal *PD-L1* in periodontitis patients and normal subjects. **(A)** Salivary exosomal *PD-L1* was measured by RT-qPCR in periodontitis patients ($n = 61$) and normal controls ($n = 30$). Error bars indicate SEM. ***$P < 0.001$ by Student's *t*-test. **(B)** Salivary exosomal *PD-L1* correlated with periodontal tissue *PD-L1* expression in periodontitis patients (Pearson's correlation test).

DISCUSSION

We characterized *PD-L1* mRNA expression in exosomes derived from saliva of periodontitis patients, and have evaluated the clinical relevance of the levels of salivary exosomes *PD-L1* mRNA in the disease. One of the main findings was that the level of salivary exosomes *PD-L1* mRNA in periodontitis patients is highly distinct relative to non-periodontitis controls. Moreover, high level of salivary exosomes *PD-L1* was associated with an advanced stage of periodontitis, suggesting it can reflect disease progression. This is the first to establish a procedure of detection of saliva-based exosomal *PD-L1* in disease, and

TABLE 2 | The clinicopathological characteristics related to *PD-L1* expression in periodontitis patients.

Variables	No. of patients	*PD-L1* expression		*P*–value*
		Low, *n* (%)	High, *n* (%)	
Total samples	61	30 (49.2)	31 (50.8)	
Age (years)				
≥50	39	18 (46.2)	21 (53.8)	0.529
<50	22	12 (54.5)	10 (45.5)	
Gender				
Female	32	19 (59.4)	13 (40.6)	0.653
Male	29	11 (37.9)	18 (62.1)	
Tobacco use				
No	40	21 (52.5)	19 (47.5)	0.474
Yes	21	9 (42.9)	12 (57.1)	
Alcohol use				
No	45	25 (55.6)	20 (44.4)	0.095
Yes	16	5 (31.3)	11 (68.8)	
Hypertension				
No	41	22 (53.7)	19(46.3)	0.316
Yes	20	8 (40.0)	12 (60.0)	
Diabetes				
No	47	25 (53.2)	22 (46.8)	0.251
Yes	14	5 (35.7)	9 (64.3)	
Stage				
Mild	26	19 (73.1)	7 (26.9)	0.005
Moderate	21	7 (33.3)	14 (66.7)	
Severe	14	4 (28.6)	10 (71.4)	

**P–values were calculated by χ^2 test. High in this analysis is based on a PD-L1 level > 9.9 (median); the remaining individuals were classified as low.*

the first salivary exosomal biomarker for periodontitis. In the majority of previous investigations of periodontitis biomarker, the specimens are either gingival tissues or gingival crevicular fluids (GCF) (Buduneli and Kinane, 2011; Ebersole et al., 2014; Jagannathan et al., 2014; Kebschull et al., 2014). However, sample collection and procedures for both gingival tissues and GCF are challenging: gingival tissue biopsy involves acquisition from invasive and limited tissue, and GCF sample collection involves sampling of a minute amount of fluid on filter paper strips, which requires a longer sampling time. Saliva-based assay overcomes these forgoing problems, as shown by our current study which involves an easy, non-invasive, and rapid collection of salivary specimens. We also demonstrate that mRNA can be extracted well from salivary exosomes, supporting the notion that exosomes-derived samples prevent mRNA from degradation (Kim et al., 2005; Soung et al., 2017). To our knowledge, this is the first to detect *PD-L1* in exosomes and in saliva in periodontitis. The procedure described in the current study may be used for detecting mRNAs in oral diseases as well as in other systemic diseases.

PD-L1 has been reported to play an important role in a wide range of cancers and inflammation-originated diseases including periodontitis (Ota et al., 2015; Clark et al., 2016; Concha-Benavente et al., 2016; Zhang et al., 2016; Groeger et al., 2017). The role of PD-L1 to inhibit destruction of inflammatory tissues has been reported (Scandiuzzi et al., 2014). In two previous reports, it has been shown that PD-L1 expression in periodontitis tissues is increased in mild and moderate periodontitis (Yuan et al., 2015; Zhang et al., 2016). However, it is unclear whether PD-L1 expression correlates with disease status of chronic periodontitis. It has been also reported that expression of PD-1 and PD-L1 in peripheral $CD4^+$ T lymphocytes and $CD8^+$ T lymphocyte of chronic periodontitis patients was upregulated (Zhu et al., 2014). High expression of PD-L1 in host cells may contribute to the chronicity of inflammatory disorders (Zamani et al., 2016). The previous work showed that the PD-L1 expression in different compartments may be different and have different roles in inflammatory disease. Therefore, further correlation between PD-L1 and periodontitis are worthy to investigated. In our current study, it suggests that assay of exosome-based *PD-L1* mRNA in saliva has potential to distinguish periodontitis from the healthy, and the levels correlate with the severity/stage of periodontitis. The increased expression of PD-L1 in severe periodontitis might due to the requirement of the body for more PD-L1 to inhibit destruction of inflammatory tissues. It has been shown that tumor cells upregulate PD-L1 expression to evade host immune response and thereby to maintain disease (Flies et al., 2016; Wang et al., 2016; Lakin et al., 2017). Moreover, the potential for PD-L1 to serve as a disease biomarker has also been revealed (Colwell, 2015; Fusi et al., 2015). PD-L1 is upregulated in cancers, such as glioblastoma (Wang et al., 2016), ovarian cancer (Abiko et al., 2013), lung cancer and oral carcinoma (Kim et al., 2005; Ota et al., 2015), and upregulated PD-L1 predicts worse survival. In periodontitis, PD-L1 has been detected in gingival tissues, gingival crevicular fluid (GCF) and blood (Olsen et al., 2016; Zhang et al., 2016; Groeger et al., 2017). Our analysis of published database validated that periodontitis specimens harbor high levels of PD-L1. Although detection of PD-L1 has been widely reported in tissues and blood (Fusi et al., 2015; Wang et al., 2016; Groeger et al., 2017), there are no publications for PD-L1 detection in salivary exosomes. We show that PD-L1 is present in exosomes of saliva from periodontitis patients, suggesting the possibility that PD-L1 is enriched in exosomes. Our results are consistent with a recent study that exosomes released from melanoma cells were found to carry a remarkable amount of PD-L1 on their surfaces (Chen et al., 2018). We here described a more convenient procedure for isolation and detection of PD-L1 in salivary exosomes. Since PD-L1 involves many diseases including cancers (i.e., oral cancers) and immune diseases (Gianchecchi et al., 2013; Dai et al., 2014; Roemer et al., 2016), the protocol established in current study may provide a platform for easy and rapid assay of PD-L1 in variety of applications. The limitation of the study was the relatively small size of the patient cohorts. Further investigations using larger and multiple cohorts are worthy to be performed to validate the findings of the current studies.

In summary, we described the procedure for isolating mRNA from exosomes derived from saliva of periodontitis patients. Our further assay showed that exosomal *PD-L1* in saliva was

enriched in periodontitis and was associated with advanced stage of disease. These findings are worthy to validate in further investigations with expanding samples.

AUTHOR CONTRIBUTIONS

HZ conceived and designed the experiments. JY and DY provided patients and the study materials. YL, KL, XX, ZY, HD, and ZJ performed the *in vitro* experiments about patient specimen analysis, bioinformatics assay, and analyzed data. JY, YL, XX, S-CY, and HZ interpreted data and wrote the manuscript. JY, S-CY, DY, and HZ contributed to discussion and reviewed the manuscript.

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