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Integrating Bench and Clinical Studies in Hepatocellular Carcinoma (HCC)

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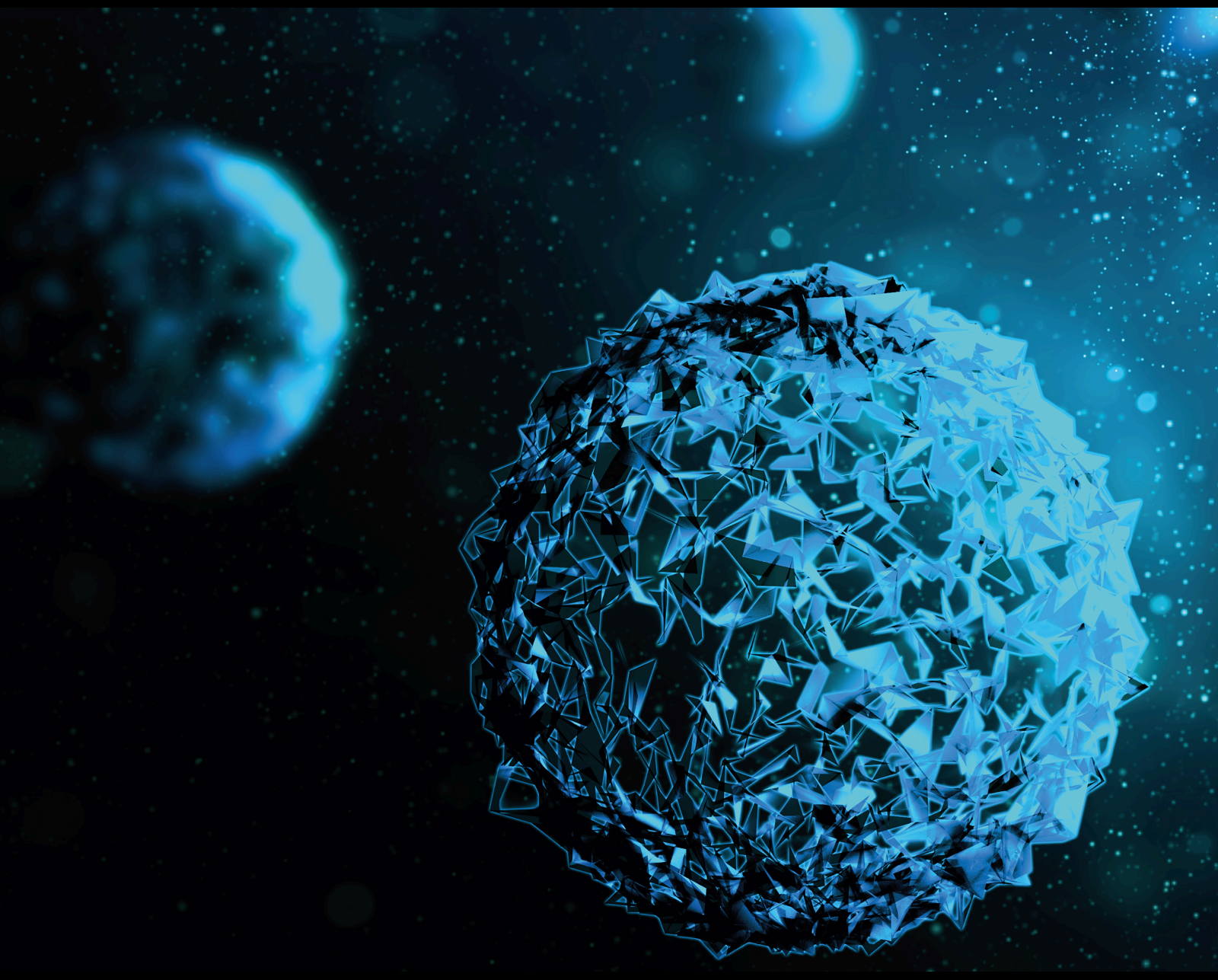

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Contents

LncRNA GSTM3TV2 Promotes Cell Proliferation and Invasion via miR-597/FOSL2 Axis in Hepatocellular Carcinoma
Yuting Hu, Wei Qiu, Zhijun Kong, Siyuan Wu, Yi Liu, Chunfu Zhu, and Xihu Qin
Research Article (10 pages), Article ID 3445970, Volume 2021 (2021)

Correlationship between Ki67, VEGF, and p53 and Hepatocellular Carcinoma Recurrence in Liver Transplant Patients
Xia Zhang, Zhixian Wu, Yonghai Peng, Dongliang Li, Yi Jiang, Fan Pan, Yi Li, Yanhua Lai, Zhongyuan Cui, and Kun Zhang
Research Article (7 pages), Article ID 6651397, Volume 2021 (2021)

COPB2: A Novel Prognostic Biomarker That Affects Progression of HCC
Jiayao Zhang, Xiaoyu Wang, Guangbing Li, Jingyi He, Ziwen Lu, Yang Yang, Yong Jiang, Liyong Jiang, Feiyu Li, and Jun Liu
Research Article (14 pages), Article ID 6648078, Volume 2021 (2021)

Intratumoral STING Agonist Injection Combined with Irreversible Electroporation Delays Tumor Growth in a Model of Hepatocarcinoma
Aritz Lasarte-Cia, Teresa Lozano, David Cano, Celia Martín-Otal, Flor Navarro, Marta Gorraiz, Noelia Casares, Isabel Vivas, and Juan José Lasarte
Research Article (9 pages), Article ID 8852233, Volume 2021 (2021)

Hepigenetics: A Review of Epigenetic Modulators and Potential Therapies in Hepatocellular Carcinoma
Mohamed H. Yousef, Hassan A. N. El-Fawal, and Anwar Abdelnaser
Review Article (30 pages), Article ID 9593254, Volume 2020 (2020)

Investigation of the miRNA and mRNA Coexpression Network and Their Prognostic Value in Hepatocellular Carcinoma
Hao Zhang, Xi Chen, and Yufeng Yuan
Research Article (19 pages), Article ID 8726567, Volume 2020 (2020)

Hindawi
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Volume 2021, Article ID 3445970, 10 pages
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Research Article

LncRNA GSTM3TV2 Promotes Cell Proliferation and Invasion via miR-597/FOSL2 Axis in Hepatocellular Carcinoma

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Mounting evidence has recently shown that role of long noncoding RNA is critical in many human cancers. lncRNA GSTM3TV2 was first proven to play a vital role in pancreatic cancer. However, the mechanism of lncRNA GSTM3TV2 in hepatocellular carcinoma (HCC) is still uncovered. Here, we object to distinguish the expression of lncRNA GSTM3TV2 and reveal its mechanistic relationship with HCC. We observed that the expression of lncRNA GSTM3TV2 and FOSL2 were upregulated in HCC. Knockdown of lncRNA GSTM3TV2 significantly inhibited cell proliferation. Meanwhile, the migration and invasion of HCC cells were greatly decreased by the downregulated lncRNA GSTM3TV2. The luciferase reporter assays showed that lncRNA GSTM3TV2 could be directly bound to miR-597, and the level of miR-597 was also decreased in the tumor tissues. lncRNA GSTM3TV2 could stabilize FOSL2 expression, resulting in the oncogenic properties of lncRNA GSTM3TV2 in HCC. Our study indicated the oncogenic activities of lncRNA GSTM3TV2 and emphasized the role of the miR-597/FOSL2 signaling pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is now becoming a main global problem, because it is one of the most mortal cancer all over the world [1]. The high mortality is due to the late stage of presentation in the majority of cases and the limited treatment options in that situation where beneficial surgery is no longer possible [2]. This late presentation is due in a large part to the absence of symptoms in the early stages of the disease and the lack of diagnostic biomarkers and other methods for detecting HCC in the early stage [3]. Potentially effective interventions, like surgery and liver transplantation, are only suitable for the early stage. The options of treatment should be loco-regional or systemic. For systemic treatment, chemotherapy and targeted therapy options are clinically approved for treating locally advanced or metastatic HCC, which extend survival by only some months in patients [4]. Given the dismal landscape of therapeutic options if diagnosis is made late, therefore, there is an urgent essential for a fine-grained molecular landscape of HCC from which to discover clinically relevant early diagnostic and prognostic biomarkers and to develop beneficial precision therapies [5].

Long noncoding RNAs (lncRNAs) are a kind of functional transcripts, whose length are more than 200 nucleotides participating in biological functions [6], including regulation of basal transcription machinery [7], gene-specific transcription [8], translation [9], and epigenetic modifications [10]. Today, increasing lcnRNAs have been reported to take part in promoting or suppressing the pathogenesis of cancer [6], including regulating cell cycle [11], survival [12], apoptosis [13], invasion, and metastasis [14] in cancers. Remarkably, some lncRNAs are found to be important roles in the efficacy of anticancer therapies in cancer, including HCC. For example, HULC could prevent miR-107 from binding E2F1 transcription factor, enhancing expression of SPHK1 and angiogenesis [15]. SNHG6-003, another upregulated lncRNA in HCC, promotes the proliferation of HCC cell lines [16]. Meanwhile, lncRNA-ATB can

inhibit the miR-200 family, which plays an important role in tumor invasion [17].

A recent study showed that GSTM3TV2 (Homo sapiens glutathione S-transferase mu 3, transcript variant 2) could promote resistance of gemcitabine in pancreatic cancer by sponging let-7 competitively to upregulate the LAT2 and OLR1 expression [18]. And the role of lncRNA GSTM3TV2 in HCC is still unknown. So, we measured the level of lncRNA GSTM3TV2 expression in HCC, and we found that lncRNA GSTM3TV2 was upregulated in HCC tissues and cell lines. We assumed that lncRNA GSTM3TV2 may play an important role in HCC and act as the ceRNA role to regulate proliferation and migration in HCC cell lines. Then, we investigated that lncRNA GSTM3TV2 could promote HCC cell proliferation and migration by upregulating FOSL2 expression through competitively sponging miR-597. Thus, the high level of GSTM3TV2 would be a worse prediction. In a word, our data revealed that GSTM3TV2 could act as a new prognostic marker and a therapeutic target in HCC.

2. Materials and Methods

2.1. Tumor Tissues Collection. 30 pairs of hepatocellular carcinoma tissues samples, as well as their adjacent normal tissues, were obtained from the Affiliated Changzhou NO.2 People's Hospital. The information was summarized in Table 1. All the pathologically and histologically HCC patients have been stored at -80°C before the study. All methods were achieved according to the guideline approved by the Ethics Committee of Affiliated Changzhou NO.2 People's Hospital. All patients were given informed consent and written informed consent was obtained.

2.2. Cell Culture. Human HCC cell lines (LO2, SMMC-7721, MHCC97-H, HepG2, and Hep3B) were cultured in DMEM medium (Invitrogen, USA), containing with 10% fetal bovine serum (Gibco, USA). All the above cells were cultured at 37°C.

2.3. Real-Time Quantitative PCR. The total RNA and miRNA Isolation was carried out by using QIAGEN Rneasy Mini kit (Invitrogen, USA) and QIAGEN miRNeasy Mini Kit (Invitrogen, USA) according to the manufacturer's instructions. Quantitative PCR analysis of miR-597 was performed by using commercial TaqMan microRNA assays (Invitrogen, USA).

2.4. MTT Assay. 4×10^3 cells were plated into 96-well plates to detect cell viabilities and cultured 18–24 h before transfecting with vectors, mimics, or plasmids. Then, 24 h, 48 h, and 72 h later, an MTT assay was performed to check the cell viabilities at 570 nm by using a Quant Universal Microplate Spectrophotometer (BioTek, USA).

2.5. Vector Construction. Complementary DNA encoding GSTM3TV2 was synthesized and subcloned into the pcDNA3.1(+) vector (Invitrogen) according to the manufacturer's instructions. The pcDNA3.1-GSTM3TV2 construct containing point mutations was synthesized by GENEWIZ (Jiangsu, China) and named pcDNA3.1-GSTM3TV2-Mut.

Table 1: Clinicopathological features of HCC patients ($n = 30$).

Patients	n (%)
Age (years)	
<60	18 (60.0)
≥60	12 (40.0)
Gender	
Male	21 (70.0)
Female	9 (30.0)
T stage	
T1-T2	11 (36.7)
T3-T4	19 (63.3)
Regional lymph node metastasis	
Yes	22 (73.3)
No	8 (26.7)
Distance metastasis	
Yes	13 (43.3)
No	17 (56.7)
Tumor size	
<5 cm	17 (56.7)
≥5 cm	13 (43.3)

The miR-597 binding region in either lncRNA-GSTM3TV2 or lncRNA-GSTM3TV2-Mut was amplified using PCR and subcloned into the pmirGLO vector (Promega, Madison, WI, USA) for use in a luciferase reporter assay.

2.6. Transwell Assay. Cell suspension was added into transwell chamber inserts (Millipore, USA) and added with matrigel. 24 h later, cells were stained and pictures were taken to measure invasion assays in accordance with the manufacturer's instructions as described previously.

2.7. Wound-Healing Migration Assay. HCC cells were cultured in six-well plates to perform migration assays. A 200 μl pipette tip was used to scratch to generate a linear gap. After 24 h, we used the microscope to take pictures and measured the width (W) of the scratch wound. The rate of close distance of the wounds was calculated. All measurements were carried out three times.

2.8. Luciferase Assay. After construction, the pGL/Luc-lncRNA GSTM3TV2-wild type or mutation plasmids were transfected into the cells (SMMC-7721 and HepG2), as well as with miR-597 mimics, and ASO (antisense oligonucleotide)-miR-597 or NC (negative control). Cell lysates were detected by a Luciferase Reporter System (Promega, USA) after 48 h.

2.9. Western Blot. The tissue and cellular protein were extracted by using RIPA lysis buffer. After centrifuge, the concentration of protein was checked by BCA kit. Western blot was performed in accordance with the standard protocol anywhere. FOSL2, Vimentin, and GAPDH antibodies were obtained from Cell Signal Technology (CST, USA). The bands were obtained from Image Lab after adding

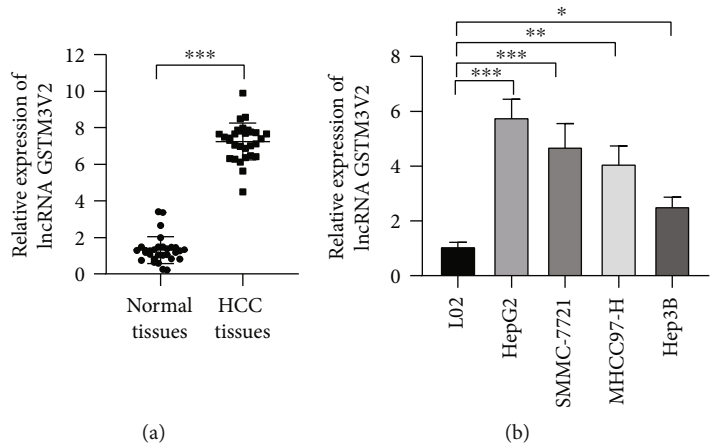


Figure 1: lncRNA GSTM3TV2 is increased in HCC tissues and HCC cell lines. (a) Real-time qPCR was performed to measure the level of lncRNA GSTM3TV2 between tumor tissues and adjacent normal tissues in HCC patients. (b) Real-time qPCR was used to identify lncRNA GSTM3TV2 level in LO2, HepG2, SMMC-7721, MHCC97-H, and HepG3B cells. Data are shown as mean ± SD. All experiments were repeated three times. $^{*}P < 0.5$; $^{**}P < 0.01$; $^{***}P < 0.001$.

chemiluminescent substrate (ECL; Millipore) to visualize. The Image Lab software was used to analyze results.

2.10. Statistical Analysis. Statistical analyses were performed by SPSS IBM 20.0. The P values were determined by using t -test (student's t-test) or ANOVA (Analysis of Variance). All data in the graphs are showed as mean ± SD. ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

3. Results

3.1. lncRNA GSTM3TV2 in HCC Was Upregulated. According to the study, it is the first time to measure the expression of lncRNA GSTM3TV2 by RT-qPCR method in 30 tumor tissues, compared with their adjacent normal tissues. Real time-PCR results indicated that lncRNA GSTM3TV2 was increased in tumor tissues significantly (Figure 1(a)). Furthermore, lncRNA GSTM3TV2 expression was detected in normal and HCC cell lines. We found that the lncRNA GSTM3TV2 expression level in HCC cell lines was about four times higher than that in normal cell lines (Figure 1(b)). The result pointed out that lncRNA GSTM3TV2 may function to be an oncogenic factor in HCC tissues and cell lines.

3.2. lncRNA GSTM3TV2 Functions as an Oncogenic Factor. Firstly, we constructed the overexpression plasmids of lncRNA GSTM3TV2 and its shRNAs to knock down its expression to investigate the expression of lncRNA GSTM3TV2 in HCC cell lines. Next, the gain-of-function and loss-of-function experiments were employed to overexpress or knock down the level of lncRNA GSTM3TV2 in HCC cell lines (HepG2 and SMMC-7721). RT-qPCR also demonstrated that the overexpression and knockdown of lncRNA GSTM3TV2 were workable (Figures 2(a) and 2(b)). Then, an MTT assay was performed to show that lncRNA GSTM3TV2 overexpression could promote the proliferation of HCC cells significantly, while decreased lncRNA GSTM3TV2 could reduce the cell viability markedly (Figures 2(c) and 2(d)). Furthermore, wound healing assay as well as transwell assay was employed to investigate whether lncRNA GSTM3TV2 could affect migration and invasion of HCC cell lines in the study. It showed that lncRNA GSTM3TV2 overexpression increased HCC cells migration and invasion significantly, while lncRNA GSTM3TV2 knockdown showed lower ability relatively (Figures 2(e) and 2(f)). Taken together, these results indicated that lncRNA GSTM3TV2 acted as an oncogenic factor, which could promote cell proliferation, migration, and invasion in HCC.

3.3. lncRNA GSTM3TV2 Sponges miR-597. More and more studies suggested that large numbers of lncRNAs can regulate the expression of the gene through acting as competing endogenous RNA (ceRNA). In order to convince whether lncRNA GSTM3TV2 could act as ceRNA to regulate gene expression, we used miRDB to predict the potential miRNA binding sites in lncRNA GSTM3TV2 (Figure 3(a)). miR-597 was chosen for further study because miR-597 is one of miRNA gaining high score. We also used luciferase assays to indicate that miR-597 could reduce luciferase activity when transfected with the wild-type lncRNA GSTM3TV2. However, miR-597 mimics did not affect the luciferase activity when cotransfected mutant lncRNA GSTM3TV2 into HCC cells (Figure 3(b)), which indicated that miR-597 could bind to lncRNA GSTM3TV2 directly. We also found that lncRNA GSTM3TV2 expression was downregulated transfecting with miR-597 mimics, while lncRNA GSTM3TV2 level was markedly upregulated after transfecting with miR-597 inhibitor (Figure 3(c)). At the same time, we detected the level of miR-597 in between HCC tissues and adjacent normal tissues, RT-PCR indicated that miR-597 was downregulated in HCC tissues (Figure 3(d)). Then, we used Pearson's correlation analysis to assess the relationship between the expression of miR-597 and lncRNA GSTM3TV2. It showed that the level of miR-597 was negative correlated

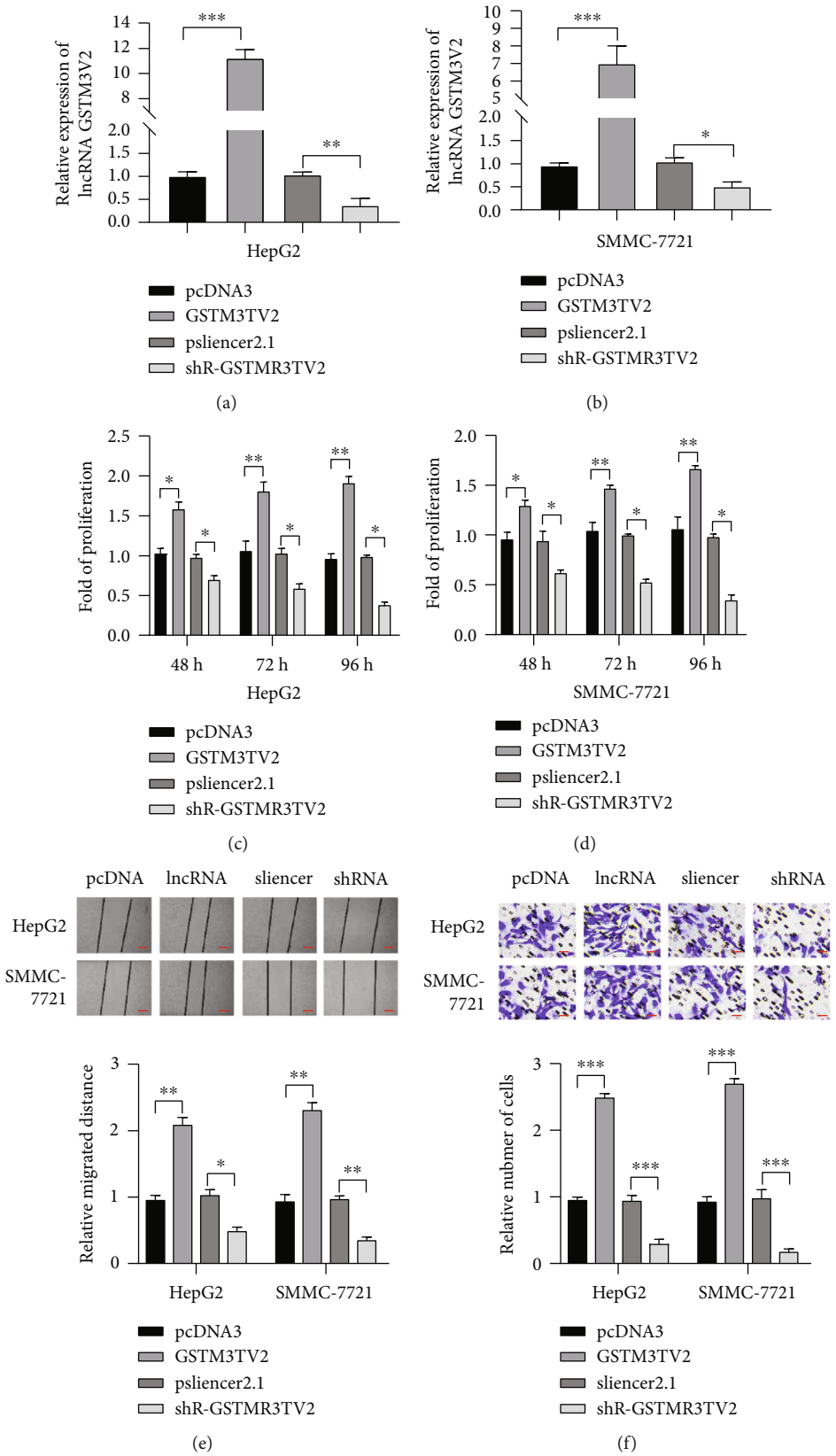
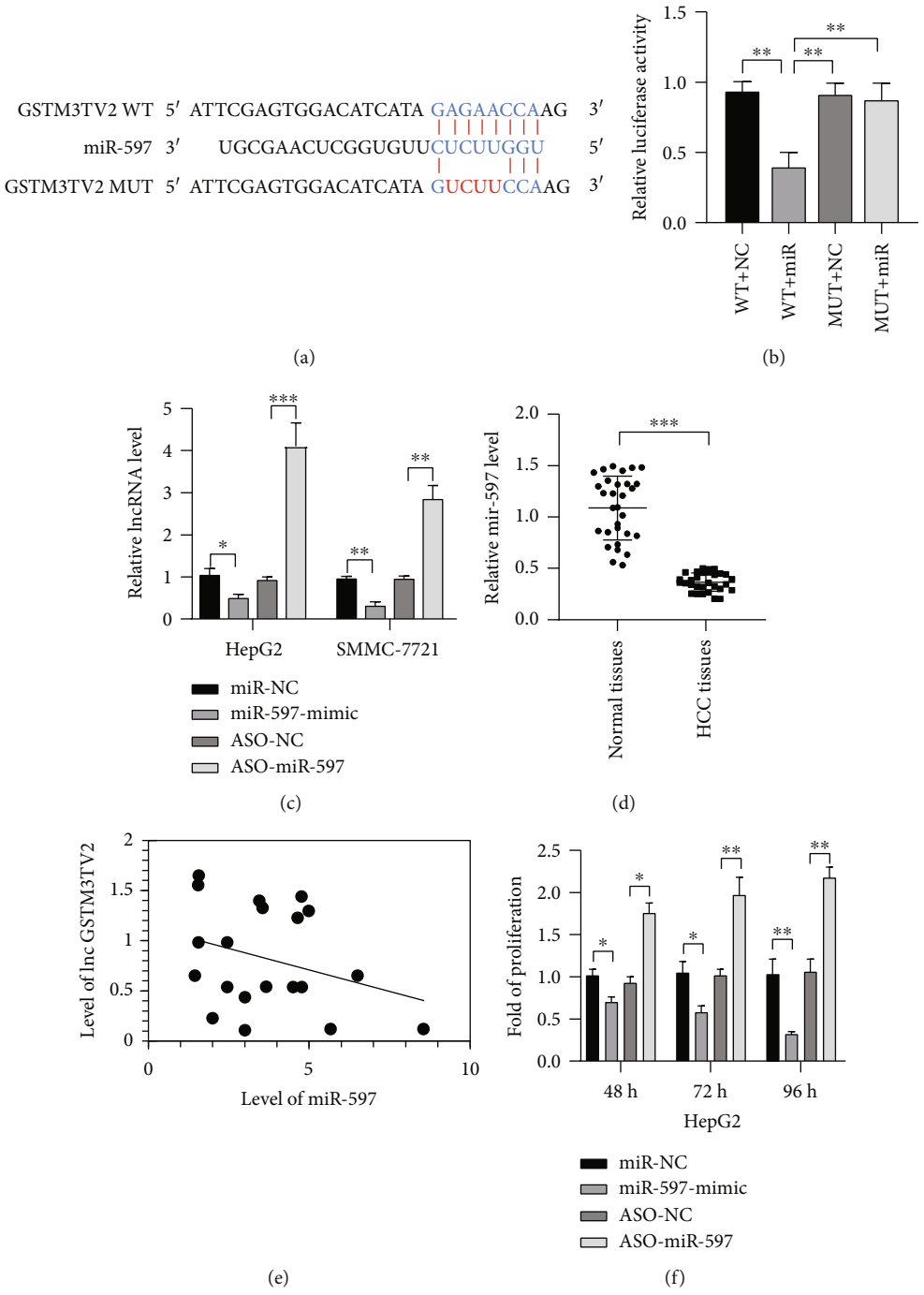


Figure 2: lncRNA GSTM3TV2 promotes proliferation, migration, and invasion in HCC cell lines. pcDNA3, pcDNA3/lncRNA GSTM3TV2, or pSilencer2.1/shR-lncRNA GSTM3TV2HCC were transfected into cell lines (HepG2 and SMMC-7721 cells). (a, b) Real-time qPCR was made to measure the level of lncRNA GSTM3TV2. (c, d) Cell viability was measured by MTT assay in HCC cell lines. (e, f) Wound healing assay and transwell assay were carried out to detect cell migration and invasion abilities. Data are shown as mean ± SD. All experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scar bar = 100 μm in wound healing assay. Scar bar = 20 μm in a transwell assay.



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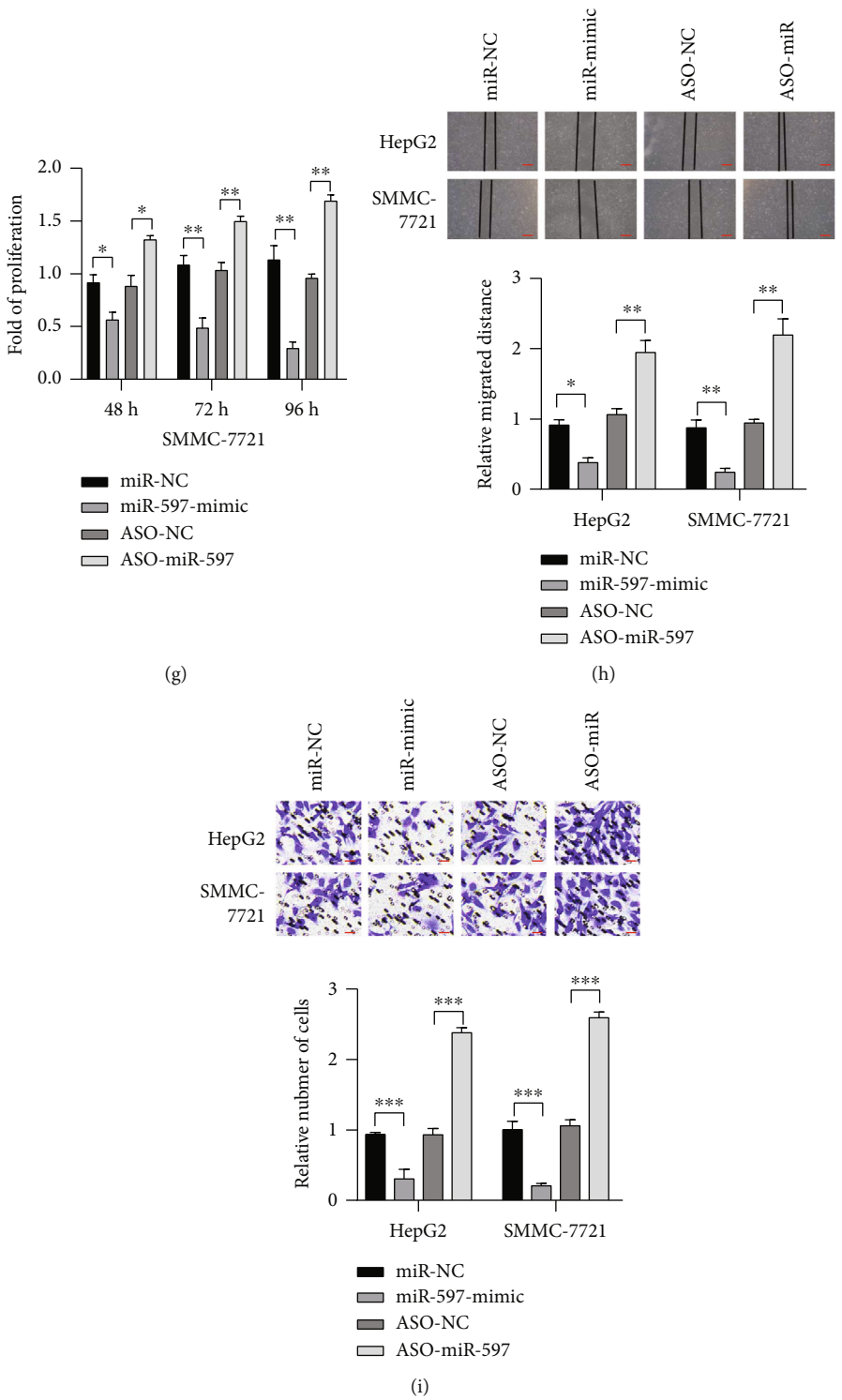


Figure 3: lncRNA GSTM3TV2 is targeted by miR-597. (a) The binding positions of miR-597 on lncRNA GSTM3TV2 were predicted by using miRDB, and the binding site of miR-597 on WT and the mutational lncRNA GSTM3TV2 were displayed. (b) Luciferase reporter system was constructed to measure the interaction between miR-597 and lncRNA GSTM3TV2. (c) The level of lncRNA GSTM3TV2 was measured by Real-time qPCR in HCC cells when transfecting miRNA mimics and ASO. (d) The level of miR-597 was measured by Real-time qPCR tumor and adjacent normal tissues in HCC patients. (e) The relationship between the expression of lncRNA GSTM3TV2 and miR-597 was analyzed by using Pearson's correlation analysis. (f, g) Cell viability was measured by MTT assay in HCC cell lines. (h, i) Wound healing assay and transwell assay were carried out to detect cell migration and invasion abilities. Data are shown as mean ± SD. All experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scar bar = 100 μm in wound healing assay. Scar bar = 20 μm in a transwell assay.

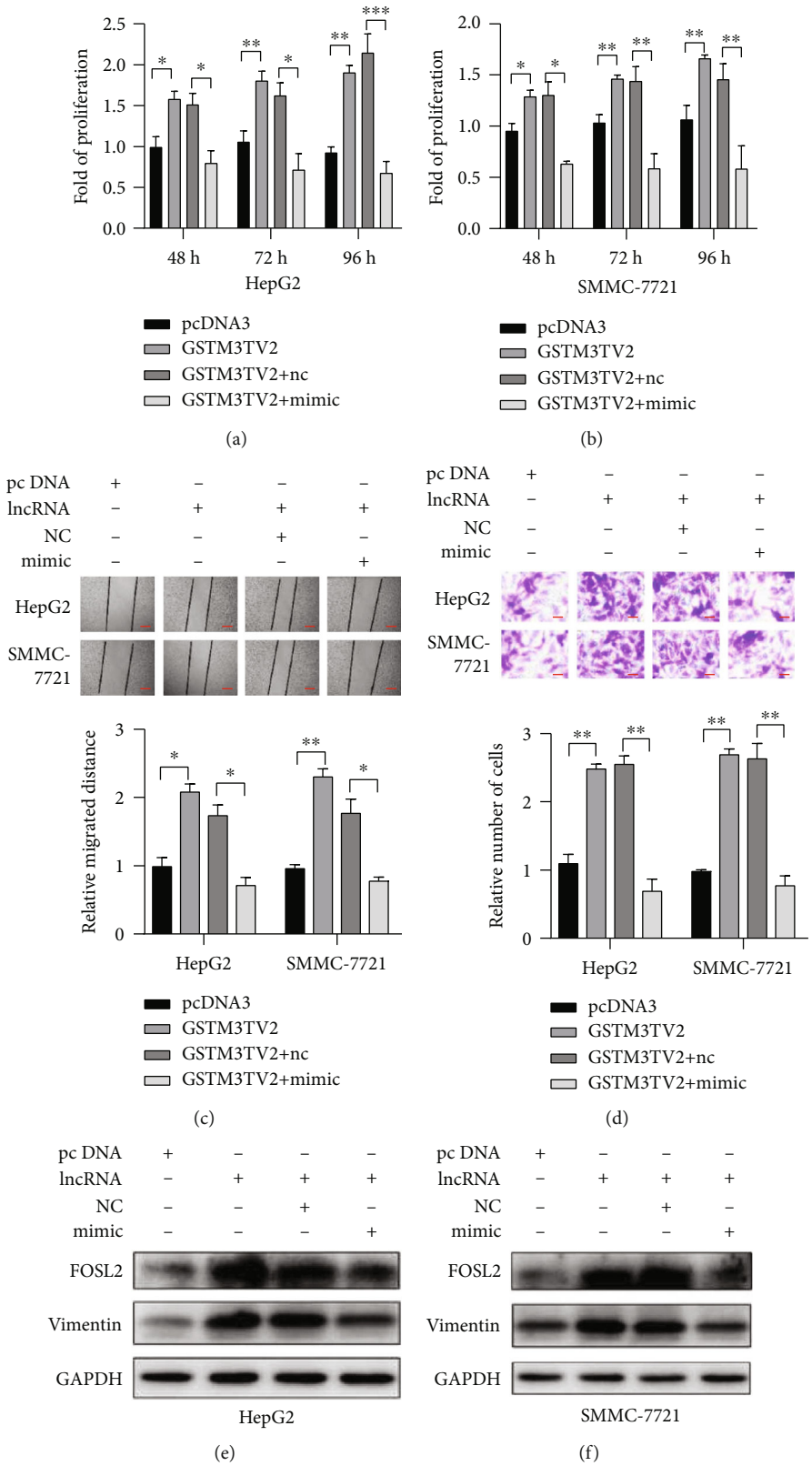
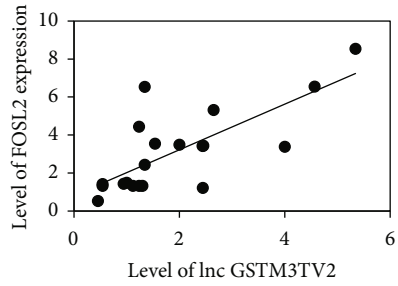


Figure 4: Continued.



(g)

Figure 4: lncRNA GSTM3TV2 rescues the tumor phenotypes caused by miR-597. pcDNA3, lncRNA GSTM3TV2, lncRNA GSTM3TV2 +miR-NC, and lncRNA GSTM3TV2+miR-597 mimics were cotransfected into HepG2 and SMMC-7721cells. (a–d) miR-597 mimics transfection could rescue viability, migration, and invasion in HCC cell lines. (e, f) FOSL2 and Vimentin protein levels were affected by lncRNA GSTM3TV2. (g) The relationship between the expression of lncRNA GSTM3TV2 and miR-597 was analyzed by using Pearson's correlation analysis. Data are shown as mean ± SD. All experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scar bar = 100 μm in wound healing assay. Scar bar = 20 μm in a transwell assay.

with lncRNA GSTM3TV2 (Figure 3(e)). Next, we detected the role of miR-597 in HCC. We transfected the miR-597 mimics and ASO-miR-597 into HCC cell lines, and the results showed that overexpression of miR-597 could decrease the proliferation, migration, and invasion of HCC cell lines (Figures 3(f)–3(i)). Taken together, we demonstrated that lncRNA GSTM3TV2 could be bound to miR-597 directly.

3.4. MiR-597 Rescues the Tumor Phenotypes of lncRNA GSTM3TV2. Then, we conducted the rescue experiments to investigate that lncRNA GSTM3TV2 played its function through the level of miR-597 in HCC cells. The wound healing assays, as well as transwell assay, showed that cotransfection with miR-597 mimics could decrease the cell viability (Figures 4(a) and 4(b)), migration (Figure 4(c)), and invasion (Figure 4(d)), which were increased by lncRNA GSTM3TV2 by in HCC cells. Furthermore, we also investigated whether lncRNA GSTM3TV2 could have effects on the expression of FOSL2, one of the targets of miR-597. The results indicated that lncRNA GSTM3TV2 overexpression increased the level of FOSL2 and Vimentin. However, cotransfection with miR-597 mimics could rescue these effects in HCC cell lines (Figures 4(e) and 4(f)). Then, we used Pearson's correlation analysis to assess the relationship between lncRNA GSTM3TV2 and FOSL2. It showed that lncRNA GSTM3TV2 was positively correlated with the level of FOSL2 (Figure 4(g)). It indicated that lncRNA GSTM3TV2 acted as a sponge for miR-597 to increase the level of FOSL2, one of the miR-597 targets, to promote HCC cells carcinogenesis.

4. Discussion

Increasing studies have been shown that lncRNAs play a critical role in lots of cancers [9]. More and more evidence also suggested that lncRNA promoted carcinogenesis in cancers, such as pancreatic cancer [19], nonsmall cell lung cancer [20], and hepatocellular carcinoma [21]. A recent study showed that lncRNA GSTM3TV2 could promote pancreatic cancer gemcitabine resistance [18]. Meanwhile, we found that lncRNA GSTM3TV2 was also overexpressed in HCC tissues and HCC cell lines. Nevertheless, the function and mechanism of lncRNA GSTM3TV2 in HCC remains to uncover. We found that lncRNA GSTM3TV2 was upregulated in tumor tissues in HCC and HCC cell lines. In our study, it is the first time to show that lncRNA GSTM3TV2 can promote proliferation, migration, and invasion in HCC cell lines, further indicate the oncogenic role of lncRNA GSTM3TV2 in HCC. Furthermore, we used miRDB to predict the potential of miRNA binding sites to investigate the exact mechanisms of lncRNA GSTM3TV2 [22]. miR-597, one of the miRNA listed, was chosen for investigating further. It is also indicated that miR-597 could function as a tumor suppressor gene in lots of cancers. For instance, miR-597 promoted HCC progression by suppressing the expression of BRMS1 [23]. Another study showed that miR-597 promoted colorectal cancer cell proliferation in vitro and in vivo by targeting FBXL2 and activating the β-catenin signaling pathway [24]. However, it was also reported that miR-597 could suppress HCC cell proliferation dependent on SMYD3 in hepatocellular carcinoma [25]. So, the role of miR-597 in cancer is controversial now. Our study showed the downregulation of miR-597 in HCC could promote HCC cell proliferation, migration, and invasion.

We also performed luciferase reporter assays, showing that lncRNA GSTM3TV2 could be directly bound to miR-597, and decreased expression of miR-597. miR-597 was downregulated in HCC tissues and cell lines, presenting a negative correlation with lncRNA GSTM3TV2. Furthermore, we demonstrated that the relation between miR-597 and FOSL2 in HCC first time. We used the database to predict the target of miR-597, one of the highest scores. As we have known, miR-597 could act as a tumor suppressor in many cancer cells, and it could downregulate the FOSL2 to suppress breast cancer cell proliferation, migration, and invasion [26]. In our study, lncRNA GSTM3TV2 could downregulate the level of endogenous miR-597, thus, increasing the expression of FOSL2, demonstrating the ceRNA function of lncRNA GSTM3TV2 through

sponging for miR-597. Thus, it is useful to detect the levels of lncRNAs, miRNAs, and other noncoding RNAs in the circulation at an early stage, and it may become a strategy to diagnose many cancers. In a word, we first demonstrate that lncRNA GSTM3TV2 is increased in tumor tissues cell lines in HCC. LncRNA GSTM3TV2 could function as an oncogene and promote HCC cell proliferation and migration by binding to miR-597, increasing the level of its target gene FOSL2. As we know, overexpression of lncRNA GSTM3TV2 increased HCC cell proliferation, migration, and invasion. Our findings indicate that lncRNA GSTM3TV2 plays an important role in HCC and can be used as a diagnostic biomarker and a target for HCC.

Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

All authors stated no potential interests to be disclosed.

Authors' Contributions

Yuting Hu and Wei Qiu are co-first authors.

Acknowledgments

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Research Article

Correlationship between Ki67, VEGF, and p53 and Hepatocellular Carcinoma Recurrence in Liver Transplant Patients

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Background and Aims. Patients with hepatocellular carcinoma (HCC) who undergo orthotopic liver transplantation (OLT) are at risk for posttransplant tumor recurrence. The aim of this study was to evaluate the correlation between the expression of Ki67, VEGF, and p53 in HCC and clinicopathological characteristics of HCC patients, as well as their predictive value for HCC recurrence after OLT. *Methods*. 60 patients who underwent OLT and were found to have HCC in the liver explant. The expression of Ki67, VEGF, and p53 in HCC was detected by immunohistochemistry. *Results*. Ki67 was associated with the tumor number and the grade of differentiation at baseline. VEGF was associated with the diameter and number of tumors, tumor differentiation, and lymph node metastasis. p53 was associated with the tumor diameter and tumor encapsulation. The expression of Ki67, VEGF, and p53 in HCC was correlated with the tumor recurrence after OLT, respectively. Among them, VEGF was an independent predictor for tumor recurrence after OLT. *Conclusion*. Ki67, VEGF, and p53 are associated with the recurrence of HCC after OLT. VEGF independently predicts the recurrence of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death globally [1, 2]. According to statistics, the annual mortality of HCC in China accounts for 55% of the global one. Because the initial symptoms of HCC are not evident, the patients are often in the intermediate or advanced stages at diagnosis. The survival of untreated HCC patients after diagnosis is usually less than 6 months [3, 4].

To date, orthotopic liver transplantation (OLT) is one of the best options in case of end-stage liver disease [5]. With the advances in medicinal treatment, five-year survival rates after OLT of over 75% have been widely observed [6, 7]. Although the survival rate is relatively high, the risk of recurrence is the major concern in transplanted patients. Clinical factors that are related to the recurrence after OLT include the size and number of tumors, micro/macrovascular invasion, and high levels of serum alpha-fetoprotein (AFP) [8].

Tumor-related factors including Ki67, VEGF, and p53 have been reported to play a role in the development and progression of HCC [9–11].

Tumor proliferating antigen (Ki67) is a nuclear protein that is recognized as a sensitive marker for cell proliferation [11]. Ki67 is highly expressed in numerous human solid tumors and is correlated with patient prognosis. Several studies found that the expression of Ki67 in numerous cancers

including prostate cancer, lung cancer, and HCC was negatively correlated with the therapeutic efficacy and prognosis [12–14]. However, the predictive value of Ki67 in the recurrence of HCC after OLT remains unclear.

Vascular endothelial growth factor (VEGF) is an important factor that mediates angiogenesis. It plays an indispensable role in tumor growth, invasion, and metastasis, as well as patient prognosis [15]. Nagoshi showed that VEGF promoted the proliferation of tumor vascular endothelial cells and served as an early marker of angiogenesis in HCC [16]. In a study of human OLT, it was suggested that the high recurrence rate after transplantation was associated with the expression of VEGF in liver grafts during the acute rejection phase [17]. Although high expression of VEGF promoted the recurrence of HCC after transplantation, whether VEGF predicts the recurrence remains unclear.

p53 is a tumor suppressor gene that regulates cell cycle and apoptosis [9]. Its mutations were found to be tumorigenic in several cancers including colon and prostate cancers, and HCC [18–20]. A study showed that the rates of gene mutation and upregulated expression of *p53* in HCC patients were 31.5% and 35.0%, respectively, and the expression of p53 was significantly associated with the poor prognosis of HCC patients [21]. However, whether p53 is associated with the recurrence of HCC after OLT has not been revealed.

Detection of factors that are associated with HCC recurrence may predict the tumor recurrence in HCC patients after OLT. In this study, we detected the expression of Ki67, VEGF, and p53 in HCC by immunohistochemical staining and explored their predictive value in tumor recurrence after OLT.

2. Materials and Method

2.1. Study Population Clinicopathological Characteristics. The study population included 60 patients who had undergone OLT at Dongfang Hospital and had complete follow-up data. All patients were diagnosed of HCC before OLT. Recurrent HCC were confirmed by a liver biopsy and pathological examination.

2.2. Clinicopathological Parameters of HCC Patients. The characteristics of HCC patients including the Child-Pugh class, the diameter and number of tumors, serum level of AFP, TNM stage, vascular invasion, liver cirrhosis, tumor encapsulation, lymph node metastasis, and tumor differentiation were collected. Patients with recurrence of HCC within 2 years were divided into the recurrence group ($n = 37$). Patients whose HCC recurred after 2 years or did not recur until the last visit were divided into the control group ($n = 23$).

2.3. Immunohistochemical Staining. Five-μm slices were obtained from paraffin-embedded specimens of tumor. Sections were dewaxed in xylene and rehydrated in alcohol followed by wet autoclave pretreatment (10 minutes at 120°C) in citrate buffer for antigen retrieval. These were rinsed in phosphate-buffered saline. Immunohistochemical staining for antibodies to Ki67 (Cat: ab15580, Abcam, Cambridge, MA), VEGF (Cat: 19003-1-AP, Proteintech, Chicago, USA), and p53 (Cat: ab1101, Abcam, Cambridge, MA) was performed using the avidin-biotin-peroxidase complex method. The primary antibody was applied to the sections and allowed to react for 25 min at room temperature. The sections were then incubated with biotinylated anti-mouse/rabbit antibody (1 : 100 dilution for Ki67, VEGF, and p53) for 25 min and avidin-biotin-peroxidase reagent for 25 min. After color development with diaminobenzimide, the sections were counterstained with hematoxylin.

2.4. Statistical Analysis. The correlation between Ki67, VEGF, and p53 and clinicopathological characteristics were analyzed by the χ^2 test and Fisher test. The correlation between Ki67, VEGF, and p53 and tumor recurrence after OLT were analyzed by single factor survival analysis and COX multivariate regression. A P value of <0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 19.0 analysis software.

3. Results

The patients were three women and 57 men with a mean age of 55 ± 15 years. 54 patients were positive for hepatitis B surface antigen (HBsAg), and six were negative. 31 patients were with a tumor diameter of <5 cm, and 29 were of 5-15 cm. The AFP level was ≥400 μg/L in 20 patients and <400 μg/L in 40. Postoperative pathological examination showed 11 cases of poorly differentiated HCC, 37 of moderately differentiated HCC, and 12 of well-differentiated HCC. The recurrence of tumors included eight cases of intrahepatic recurrence, seven of intrahepatic and extrahepatic recurrence, 18 of lung metastasis, nine of bone metastasis, six of lymph node metastasis, and three of brain metastasis.

3.1. Correlationship between Ki67, VEGF, and p53 and Clinicopathological Characteristics of HCC Patients. The expression of Ki67 was correlated with the number of tumors ($P = 0.005$) and the grade of tumor differentiation ($P = 0.038$). However, it was not associated with other clinicopathological characteristics of HCC patients (Table 1). The expression of VEGF was associated with the diameter and number of tumors ($P = 0.037$ and $P = 0.005$), tumor differentiation ($P = 0.035$), and lymph node metastasis ($P = 0.025$), whereas it was not related to other clinicopathological characteristics (Table 1). The expression of p53 was correlated with the tumor diameter ($P = 0.044$) and tumor encapsulation ($P = 0.022$). There was no correlation between p53 and other clinicopathological characteristics (Table 1).

3.2. The Expression of Ki67, VEGF, and p53 in HCC. In the postoperative HCC samples from 37 patients in the recurrence group and 23 in the control group, the positive expression rates of Ki67 were 67.5% (25/37) and 39.1% (9/23), respectively (Figures 1(a), 1(b), and 2). The positive expression rates of VEGF were 56.7% (21/37) and 30.4% (7/23), respectively (Figures 3(a), 3(b), and 2). The positive expression rates of p53 were 62.1% (23/37) and 34.7% (8/23), respectively (Figures 4(a), 4(b), and 2). Logistic single-factor statistical analysis showed that the positive expression rates of Ki67, VEGF, and p53 were different between the

Table 1: Correlation between the expression of Ki67, VEGF, and p53 and clinicopathological characteristics of HCC patients.

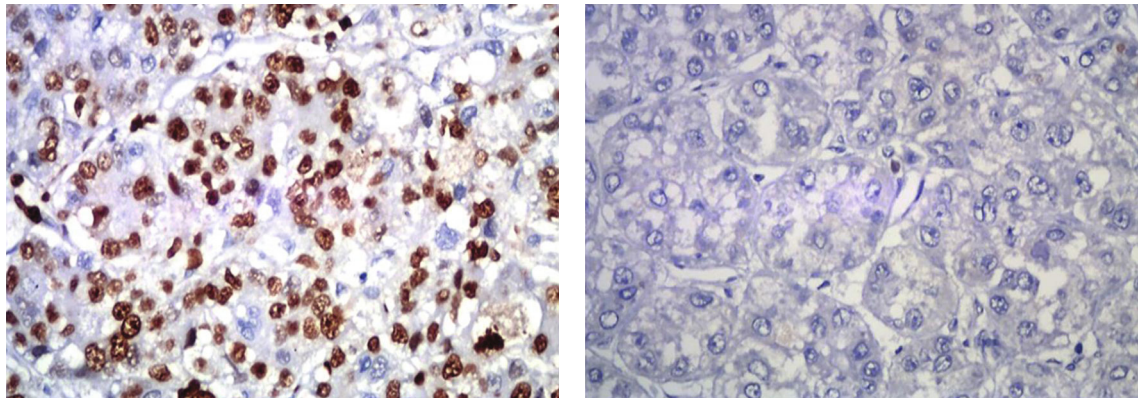
Clinicopathological characteristics		Ki67 expression			VEGF expression			p53 expression		
		-	+	*P*	-	+	*P*	-	+	*P*
Gender				1.000*			1.000*			0.238*
	Male	25	32		30	27		29	28	
	Female	2	1		2	1		0	3	
Age				0.388*			0.088*			1.000*
	≦60	22	32		31	23		26	28	
	>60	4	2		1	5		3	3	
Child-Pugh class				1.000*			0.883*			0.494*
	A	8	11		10	9		8	11	
	B	18	12		22	18		21	19	
	C	0	1		0	1		0	1	
Tumor size				0.203†			0.037†			0.044†
	≦5.0	16	15		21	10		19	12	
	>5.0	10	19		11	18		10	19	
Tumor number				0.005†			0.005†			0.617†
	1	19	12		11	20		16	15	
	≧2	7	22		21	8		13	16	
AFP				0.174†			1.000†			0.419†
	>400	6	14		11	9		8	12	
	≦400	20	20		21	19		21	19	
TNM staging				0.122*			0.659*			0.857*
	I	4	2		4	2		3	3	
	II	11	9		12	8		4	11	
	III	6	3		3	6		9	5	
	IV	5	20		13	12		13	12	
Vascular invasion				0.398*			0.783†			0.544†
	Yes	4	14		6	12		7	11	
	No	22	20		26	16		22	20	
Cirrhosis				0.072*			0.379†			0.082*
	Yes	23	23		23	23		25	21	
	No	3	11		9	5		4	10	
Tumor encapsulation				0.416†			0.578†			0.022*
	Yes	21	21		24	19		25	18	
	No	5	12		8	9		4	13	
Lymphatic metastasis				1.000*			0.025*			0.750*
	Yes	1	1		0	2		0	2	
	No	25	33		32	26		29	29	
Differentiation				0.038*			0.035*			0.081*
	Low	1	10		2	9		2	9	
	Medium	19	18		22	15		21	37	
	High	6	6		8	4		6	12	

∗: Fisher test; †: Pearson chi-square test; "+": positive; "-": negative.

recurrence group and the control group, respectively ($P = 0.015$, 0.008, and 0.035, Table 2).

3.3. Correlationship between the Expression of Ki67, VEGF, and p53 and the Recurrence of HCC after OLT. Single-factor survival analysis showed that the positive expression of Ki67 (Log Rank $P = 0.036$ and Breslow $P = 0.047$), the positive expression of VEGF($P = 0.003$ and $P = 0.001$), and the positive expression of p53 ($P = 0.015$ and $P = 0.011$) were associated with tumor recurrence, respectively (Table 3, Figures 5(a)–5(c)).

As analyzed by multivariate regression of COX, the diameter and number of tumors ($P = 0.016$), the tumor



(a) (b)

Figure 1: (a) Positive immunostaining with nuclear Ki67 expression in HCC. (b) Negative Ki67 expression with low nuclear reactivity in HCC.

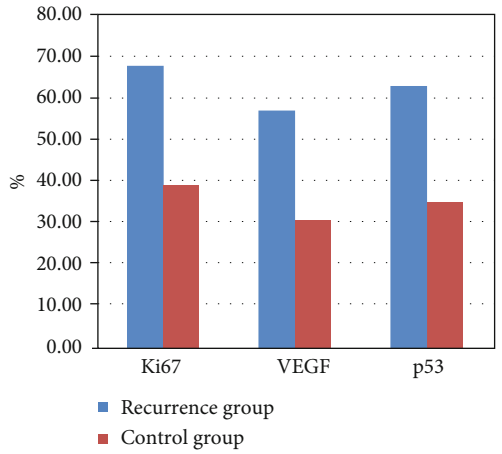


Figure 2: The positive expression rates of Ki67, VEGF, and p53 in the recurrence group and the control group.

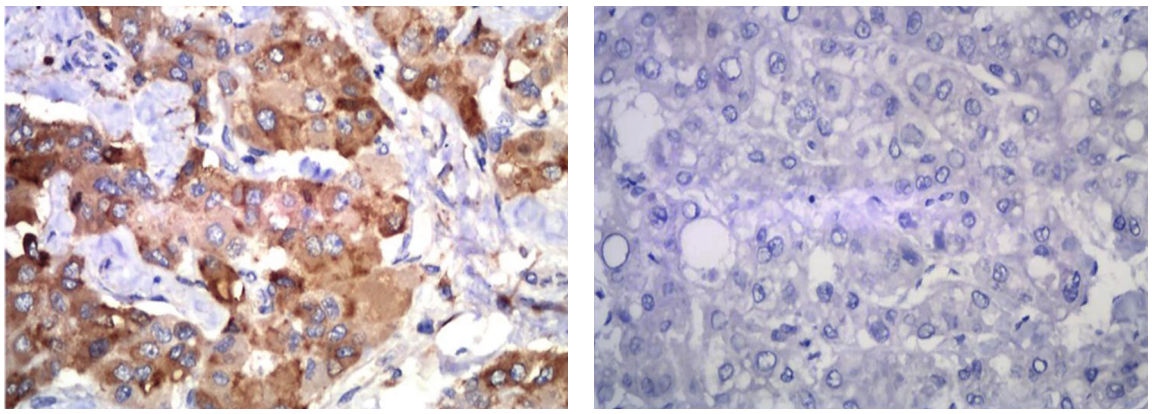
encapsulation ($P = 0.022$), the vascular invasion ($P = 0.009$), the TNM stage ($P = 0.036$), and the positive expression of VEGF ($P = 0.005$) in HCC were associated with the HCC recurrence after OLT. However, the positive expression of Ki67 ($P = 0.142$) and p53 ($P = 0.062$) did not predict the tumor recurrence and metastasis (Table 4).

4. Discussion

The recurrence of HCC gives rise to an unsatisfactory survival rate of HCC patients after OLT. Several studies suggested that the causes of HCC recurrence after OLT might include the following [22–25]: (1) Micrometastasis of cancer cells had occurred before transplantation. (2) Handling the diseased liver during the operation caused tumor rupture and thereby iatrogenic cancer metastasis. (3) The use of immunosuppressants promoted the proliferation and invasion of tumor cells. Recent studies showed that tumor cells and several biological molecules secreted by the microenvironment of tumors play an important role in the recurrence of tumors [26]. Preoperative detection of these molecular markers and postoperative quantitative assays of corresponding antibodies may predict tumor recurrence after OLT.

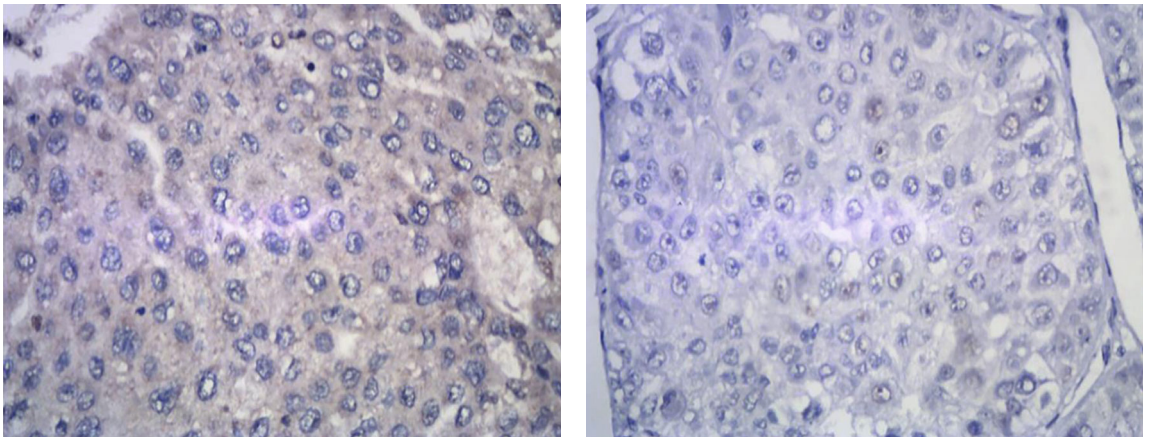
The current study showed that the expression of Ki67 in HCC was correlated with the number of tumors and the grade of tumor differentiation in HCC patients whereas it was not associated with other clinicopathological characteristics. It is suggested that the expression of Ki67 is related to the proliferation and malignant biological activities of liver cancer cells. In addition, the positive expression rate of Ki67 was higher in the recurrence group than the control group. The patients with positive expression of Ki67 had worse disease-free survival after surgery. It is suggested that HCC with high expression of Ki67 is prone to invasion and metastasis. However, COX multivariate survival analysis indicated that Ki67 had no independent predictive value for tumor recurrence after OLT. We infer that Ki67 is not specific to malignant tumors, and its expression may be affected by other factors, such as nutrient supply to cells. Therefore, Ki67 may be useful in predicting the prognosis when combined with other indicators.

VEGF is an essential factor in tumor growth, which plays a role in tumor growth and invasion, and patients prognosis [17]. The increased level of VEGF is mainly secreted by an autocrine or a paracrine measure by hepatic stellate cells and tumor cells [25]. Jeng et al. found that the expression of *VEGF* mRNA in HCC patients with the portal vein tumor was significantly higher than those without, and multivariate analysis also showed that the expression of VEGF was correlated with the portal vein thrombosis [27]. In the current study, the expression of VEGF in HCC was correlated with the diameter and number of tumors, tumor differentiation, and lymph node metastasis, which is consistent with the study by Jia et al. [28]. It is indicated that VEGF is involved in the development and progression of HCC. In addition, the positive expression rate of VEGF in the recurrence group was higher than the control group and the expression of VEGF was negatively correlated with the tumor-free survival. Therefore, VEGF is a critical risk factor for HCC recurrence after OLT and is an independent predictor of tumor recurrence after OLT in HCC patients.



(a) (b)

Figure 3: (a) Positive immunostaining with VEGF expression in HCC. (b) Negative VEGF expression immunostaining in HCC.



(a) (b)

Figure 4: (a) Positive immunostaining with p53 expression in HCC. (b) Negative p53 expression immunostaining in HCC.

Table 2: The expression of Ki67, VEGF, and p53 in the recurrence group and the control groups.

Name	Expression	Relapse group	Control group	95% CI*	*P* value
Ki67	Negative	12	14	(0.288, 3.718)	0.015
	Positive	25	9		
VEGF	Negative	16	16	(0.404, 3.588)	0.008
	Positive	21	7		
p53	Negative	14	15	(0.043, 2.823)	0.035
	Positive	23	14		

∗: logistic analysis.

Table 3: Correlation between Ki67, VEGF, and p53 expression and tumor recurrence.

Name	χ^2	Log Rank *P* value	χ^2	Breslow *P* value
Ki67	4.404	0.036	3.936	0.047
VEGF	8.807	0.003	11.614	0.001
p53	5.947	0.015	6.445	0.011

Note: Kaplan-Meier analysis with two analytical methods.

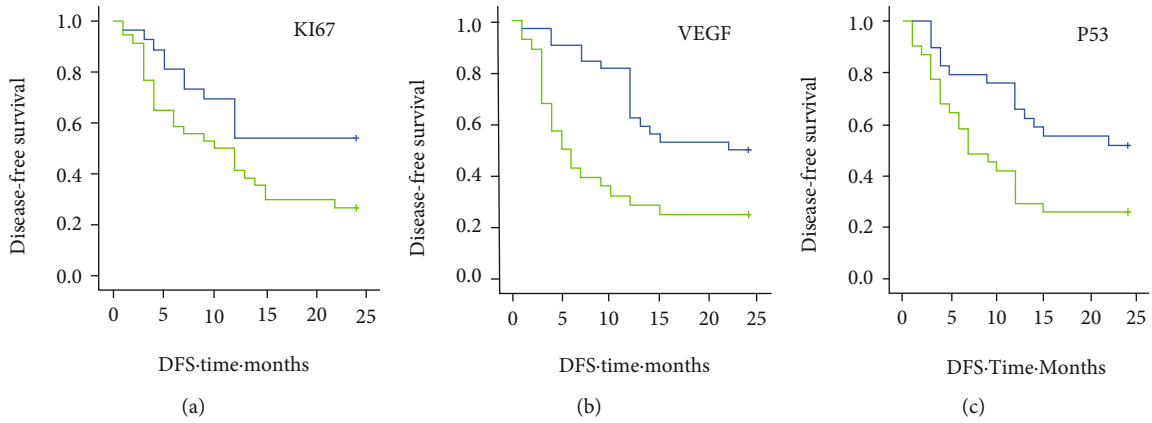


Figure 5: (a) Disease-free survival (DFS) of patients with negative and positive expression of Ki67 expression; blue line: negative; light green line: positive; Log Rank $P = 0.036$; Breslow $P = 0.047$; (b) disease-free survival (DFS) of patients with negative and positive expression of VEGF. Blue line: negative; light green line: positive; Log Rank $P = 0.003$; Breslow $P = 0.001$. (c) Disease-free survival (DFS) of patients with negative and positive expression of p53, Log Rank $P = 0.015$; Breslow $P = 0.011$.

Table 4: Correlation between the expression of Ki67, VEGF, and p53 and recurrence of HCC after OLT.

	β	S.E	Wald χ^2	P value	OR	95% CI*
Ki67	0.642	0.438	2.153	0.142	1.901	(-0.513, 1.872)
VEGF	1.387	0.492	7.942	0.005	4.004	(0.346, 3.265)
p53	0.801	0.430	3.474	0.062	2.228	(-0.147, 2.101)

*: COX multifactor regression analysis.

In this study, p53 expression was associated with tumor encapsulation and tumor diameter, but not with the age, serum AFP level, number of tumors, tumor differentiation, and TNM stage ($P > 0.05$). It indicates that the proliferation and invasion of liver cancer cells after OLT are related to the expression of p53. Additionally, the positive expression rate of p53 was high in the recurrence group and it was associated with tumor recurrence. However, it was showed that the positive expression of p53 did not exhibit predictive value in tumor recurrence after OLT in HCC patients.

There were several limitations regarding the study. It was a retrospective study and had a relatively small sample. Only the correlationship between markers and clinicopathological characteristics were demonstrated but no in-depth mechanisms were revealed. Future studies with a larger cohort of patient samples will be needed to further support the findings.

5. Conclusion

In conclusion, Ki67, VEGF, and p53 are associated with the recurrence of HCC patients after OLT. Nevertheless, only VEGF independently predicts the recurrence of HCC patients after OLT. It is necessary to identify robust predictors of HCC recurrence after OLT, which facilitates the screening of patients with a high risk of HCC recurrence.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions

Xia Zhang and Zhixian Wu contributed equally to this work.

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Research Article

COPB2: A Novel Prognostic Biomarker That Affects Progression of HCC

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Purpose. This study is aimed at investigating the expression, underlying biological function, and clinical significance of coatomer protein complex subunit beta 2 (COPB2) in hepatocellular carcinoma (HCC). *Methods*. HCC-related data were extracted from The Cancer Genome Atlas (TCGA) database, International Cancer Genome Consortium (ICGC) database, and Gene Expression Omnibus (GEO) database. A logistic regression module was applied to analyze the relationship between the expression of COPB2 and clinicopathologic characteristics. The Cox proportional hazard regression model and Kaplan–Meier method were used for survival analysis. Gene set enrichment analysis (GSEA) was used to annotate the underlying biological functions. Loss-of-function experiments were conducted to determine the underlying mechanisms. *Results*. COPB2 was overexpressed in HCC, and high expression of COPB2 was significantly correlated with higher alpha fetoprotein (AFP) (odds ratio (OR) = 1.616, >20 vs. ≤20, $p < 0.05$), stage (OR = 1.744, III vs. I, $p < 0.05$), and grade (OR = 1.746, G4+G3 vs. G2+G1, $p < 0.05$). Kaplan–Meier survival analysis showed that HCC patients with high COPB2 expression had a worse prognosis than those with low COPB2 expression ($p < 0.0001$ for TCGA cohort, $p < 0.05$ for ICGC cohort). The univariate Cox (hazard ratio (HR) = 1.068, $p < 0.0001$) and multivariate Cox (HR = 2.011, $p < 0.05$) regression analyses suggested that COPB2 was an independent risk factor. GSEA showed that mTOR and other tumor-related signaling pathways were differentially enriched in the high COPB2 expression phenotype. Silencing of COPB2 inhibited the proliferation, migration, and invasion abilities by suppressing epithelial-mesenchymal transition and mTOR signaling. *Conclusion*. COPB2 is a novel prognostic biomarker and a promising therapeutic target for HCC.

1. Introduction

Liver cancer is one of the most common fatal cancers, ranking sixth among cancer diagnoses, and is the fourth leading cause of cancer-related deaths, with 841,000 new cases and 782,000 deaths annually worldwide [1]. The morbidity and mortality of liver cancer rank fourth (10.6%) and third (12.9%), respectively, among all malignant tumors in China [2]. Hepatocellular carcinoma (HCC) accounts for approximately 80% of primary liver cancers; due to its asymptomatic disease progression and limited treatment options, it has become a leading cause of cancer burden globally [1, 3, 4]. There are several ways to treat HCC, such as surgical resection, locoregional therapy, liver transplantation, and systemic therapy; however, its prognosis remains poor, and its survival rate is much lower for patients with metastasis and recurrence [5–8]. Therefore, finding new biomarkers is very important for the prognosis and treatment of HCC and will benefit more patients.

The coatomer protein complex subunit beta 2 (COPB2), encoded by a gene located on chromosome 3q23 [9], is one of the seven subunits that form coatomer complex I (COPI), which is one of the three types of coat proteins (COPs) that play a key role in intracellular transport by forming transport

vesicles [10]. Previous studies have reported that the main functions of COPB2 are the regulation of extracellular membrane transport and mediation of retrograde transport from the Golgi complex to the endoplasmic reticulum (ER) [11–13]. Recently, COPB2 was reported to have important correlations with various cancer types and has different functions in different tumors, such as breast cancer, glioma, and prostate cancer [14–16]. Silencing COPB2 can inhibit the proliferation of colon cancer cells by inducing cell cycle arrest [17]. In addition, coatomer protein complex subunit alpha (COPA), another subunit of COPI, is an important paralog of COPB2 [18] and has been reported to be upregulated in tumors relative to paired adjacent nonmalignant tissues in patients with liver cancer [19]. It was also reported that reduced editing of *COPA* was implicated in the pathogenesis of HCC and editing of $COPA^{WT}$ may switch it from a tumor-promoting gene to a tumor suppressor by deactivating the PI3K/AKT/mTOR pathway through downregulation of caveolin-1 (CAV1) [20]. Deregulated mTOR signaling significantly contributes to the molecular pathogenesis of HCC [21]. Considering the relationship between COPB2 and other tumors and the role of its paralog, COPA, in HCC, we hypothesized that COPB2 may play an important role in the progression of HCC and may be a new potential therapeutic target for HCC. By querying the online UALCAN database (http://ualcan.path.uab.edu/analysis.html), we found that COPB2 expression was elevated in HCC and correlated with its prognosis [22], but no research has yet revealed the mechanism by which COPB2 regulates the malignant progression of HCC.

In this study, we explored the role of COPB2 in HCC by analyzing HCC-related data from The Cancer Genome Atlas (TCGA) database, the International Cancer Genome Consortium (ICGC) database, and Gene Expression Omnibus (GEO) databases, as well as conducting a series of experiments. The results of the current study revealed that COPB2 is a novel prognostic biomarker and a promising therapeutic target for HCC.

2. Material and Methods

2.1. Bioinformatics Analysis. All HCC-related data (including clinical information and corresponding mRNA expression data) were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/repository) and the International Cancer Genome Consortium (ICGC) database (https://dcc.icgc.org/releases). We then used Perl (v 5.26.3) and R (v 3.6.3) to sort and extract the data and merged the expression data with clinical data. Cases without clinical data or expression data were excluded. At the same time, patients with a pathological type other than primary hepatocellular carcinoma were also excluded; 370 HCC cases from TCGA cohort and 232 HCC cases from the ICGC cohort were used for subsequent analysis. The patients' basic information is shown in Tables S1 and S2. The clinical characteristics of TCGA cohort included age, sex, body mass index (BMI), T stage, lymph node (N), metastasis (M), stage, grade, tumor status, family history of cancer, vascular invasion, AFP, new tumor event, survival status, and survival time, while the ICGC cohort included age, sex, stage, grade, and tumor status. Cases with incomplete clinical pathological information were included in the analysis based on the available clinical information and excluded from the analysis of the clinical pathological features where data were missing. In the survival analysis, patients with a survival time of less than 30 days were excluded, since they may have died of serious complications (including bleeding, intracranial infections, and heart failure) rather than HCC. To further verify the expression level of COPB2 mRNA in patients with HCC, six datasets from the Gene Expression Omnibus (GEO) database were used (Table S3). In the present study, in addition to difference analysis and Kaplan–Meier analysis, the logistic regression and Cox proportional hazard regression models were used for clinical correlation analysis and survival analysis, respectively. Gene set enrichment analysis (GSEA) is a method to identify classes of genes or proteins that are overrepresented in a large set of genes or proteins and may be associated with disease phenotypes [23]. GSEA was used to explore the potential biological signaling pathways related to COPB2 in HCC. During each analysis, all genes were generated in an ordered list and were classified into high and low COPB2 expression phenotypes. Gene set permutations were performed 1000 times. A nominal p value < 0.05 and false discovery rate $(\mathrm{FDR}) < 0.05$ were used to filter the pathways enriched in each phenotype.

2.2. Cell Culture and siRNA Transfection. BEL7402 and SMMC7721 HCC cell lines were purchased from the BeNa Culture Collection (Beijing, China). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified chamber with 5% CO_2 at 37°C. siRNA for COPB2 was purchased from Genomeditech (Shanghai, China). BEL7402 and SMMC7721 cells were seeded in six-well plates at 30–50% confluence and were then transfected with 50 nmol/L siRNAs using Lipofectamine 3000 reagent (Thermo, L3000015, Waltham, MA, USA). The cells were collected following transfection efficiency determination and follow-up experiments after being transfected for 48–72 hours.

2.3. CCK-8 Assay. The transfected BEL7402 and SMMC7721 cells were seeded and cultured in four 96-well plates at 2000 cells/well, with five replicate wells for each group; the cell viability of each group was measured after 0 h, 24 h, 48 h, and 72 h at a wavelength of 450 nm with a microplate reader after adding CCK-8 reagent for 3 h in each well.

2.4. Immunohistochemistry. Tumor tissues and corresponding adjacent nontumor tissues in 20 HCC patients undergoing hepatectomy were fixed with 4% paraformaldehyde immediately after isolation and then embedded in paraffin for being cut into 5 μm thick continuous sections. These sections were then deparaffinized, hydrated, and incubated overnight with the primary rabbit anti-COPB2 polyclonal antibody (Abcam, ab192924, CA, USA) and primary rabbit anti-phospho-mTOR (Ser2448) polyclonal antibody (CST, 2796, MA, USA) overnight at 4°C. On the next day, the cells were incubated with the secondary antibody at 37°C and then

visualized using a DAB kit (ZSGB-BIO, ZLI-9017, Beijing, China) and counterstained with hematoxylin. The expression level was independently evaluated by two senior pathologists using the H-score method.

2.5. Scratch Wound Healing Assay. The transfected BEL7402 and SMMC7721 cells were seeded in six-well plates. When they reached approximately 80–90% confluence, the cells were scratched using a 200 μL pipette tip. Serum-free medium was added after washing with phosphate-buffered saline to remove debris. Photographs were taken at 0 h and 48 h to compare wound healing rates.

2.6. Transwell Assay. For the migration assay, 4×10^4 cells (200 μL serum-free cell suspension) were seeded into the upper Transwell chamber with 8 μm pore inserts (Corning, NY, USA), while the bottom chamber was filled with 600 μL RPMI 1640 medium supplemented with 10% fetal bovine serum. After incubation at 37°C with 5% CO_2 for 24 h, cells invading the lower surfaces were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet stain solution, while cells on the upper surface were scraped. Nine random fields were used for statistical analysis. For the invasion assay, 1×10^5 cells (200 μL serum-free cell suspension) were seeded into the upper Transwell chamber, which was prepaved with Matrigel. The remaining steps were the same as those for the migration assay.

2.7. Cell Cycle Distribution. All cells were collected, fixed, and stained after being transfected for 72 h, and the cell cycle distribution was detected using a Muse Cell Analyzer (Merck & Millipore, Germany). All experimental procedures were performed in accordance with the manufacturer's protocol.

2.8. Western Blotting. The total protein in each group of cells was lysed in PIPA lysis buffer (Solarbio, R0010) supplemented with phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Thermo Scientific, 36987, Waltham, MA, USA) and phosphatase inhibitor (Thermo Scientific, 78428, Waltham, MA, USA); and their concentrations were then measured using a BCA Protein Assay Kit (Solarbio, PC0020, Beijing, China). 30 μg/well of protein extracts was separated on 10% SDS-PAGE and transferred onto Nitrocellulose Transfer Membrane (PALL, 66485, NY, USA). After cutting into different strips according to the molecular weight of target proteins, the membranes were reacted with primary antibodies against target proteins overnight on a shaker at 4°C. On the next day, these bands were visualized after incubation with the secondary antibody.

2.9. Statistical Analysis. GraphPad Prism 8.0, SPSS 25.0, and R 3.6.3 software were used for all statistical analyses. The distribution of all data was tested for normality prior to statistical analysis. When comparing the differences between two groups, we used the *t*-test for normally distributed data and used a nonparametric test (unpaired: Mann–Whitney *U* test; paired: Wilcoxon matched-pairs signed rank-test) for the data that were not normally distributed. The data of CCK-8 assays was analyzed using two-way repeated measurement ANOVA with Sidak's multiple comparisons test. Survival was analyzed using a Kaplan–Meier plot and log-rank test. The correlation analysis between COPB2 expression level and clinicopathological parameters in HCC patients used logistic regression. The correlation between different clinicopathological variables and overall survival was explored using univariate and multivariate Cox proportional hazard regression mode. $p < 0.05$ was considered to be a significant statistical difference.

3. Results

3.1. COPB2 Overexpressed in HCC. The mRNA expression data of 370 HCC tissues and 50 matched nontumor tissues from TCGA cohort were analyzed. The results showed that COPB2 mRNA was significantly overexpressed in HCC tissues compared with the expression in nontumor tissues using unpaired and paired tests (Figure 1(a); unpaired: $p < 0.0001$, paired: $p < 0.0001$). For the ICGC cohort, 232 HCC tissues and 199 matched nontumor tissues were analyzed, and the results were consistent with those of TCGA cohort (Figure 1(b); unpaired: $p < 0.0001$, paired: $p < 0.0001$). To further verify the expression level of COPB2 mRNA in patients with HCC, six datasets from the GEO database were analyzed and similar results were obtained: GES76427 (tumor = 115, nontumor = 52) (Figure 1(c); unpaired: $p < 0.0001$, paired: $p < 0.0001$), GSE14520 (tumor = 225, nontumor = 220) (Figure 1(d); unpaired: $p < 0.0001$, paired: $p < 0.0001$), GSE39791 (tumor = 72, nontumor = 72) (Figure S1A; unpaired: $p < 0.0001$, paired: $p < 0.0001$), GES36411 (tumor = 42, nontumor = 42) (Figure S1B; unpaired: $p < 0.001$), GSE102079 (tumor = 152, nontumor = 105) (Figure S1C; unpaired: $p < 0.01$), and GSE25097 (tumor = 268, nontumor = 289) (Figure S1D; unpaired: $p < 0.001$). In order to verify the results of the above bioinformatics analysis, we performed immunohistochemical staining on tumor tissues ($n = 20$) and matched nontumor tissues ($n = 20$) from HCC patients; as expected, the results showed that COPB2 was significantly overexpressed in tumor tissues (Figures 1(e) and 1(f), $p < 0.0001$).

3.2. High COPB2 Expression Was Correlated with Poor Prognosis in HCC Patients. We conducted a further correlation analysis on the expression data and clinical data of HCC cases from TCGA and ICGC databases. The results indicated that high expression levels of COPB2 positively correlated with the clinical characteristics of poor prognosis. There were significant differences in COPB2 expression between different subgroups defined based on AFP (≤20 ($n = 147$) vs. >20 ($n = 130$), $p < 0.05$), T stage (T2 ($n = 93$) vs. T1 ($n = 181$), $p < 0.05$; T3 ($n = 80$) vs. T1 ($n = 181$), $p < 0.05$), stage (Stage III ($n = 85$) vs. Stage I ($n = 171$), $p < 0.01$), and grade (G3 ($n = 121$) vs. G1 ($n = 55$), $p < 0.05$; G3 ($n = 121$) vs. G2 ($n = 177$), $p < 0.01$) in TCGA cohort (Figures 2(a)–2(d)) and stage (Stage IV ($n = 19$) vs. Stage I ($n = 36$), $p < 0.01$; Stage IV ($n = 19$) vs. Stage II ($n = 106$), $p < 0.05$; Stage III ($n = 71$) vs. Stage I ($n = 36$), $p < 0.05$) and grade (G3 ($n = 58$) vs. G1 ($n = 32$), $p < 0.001$; G2 ($n = 121$) vs. G1 ($n = 32$), $p < 0.05$) in the ICGC cohort (Figures 2(e) and 2(f)). Meanwhile, a logistic regression

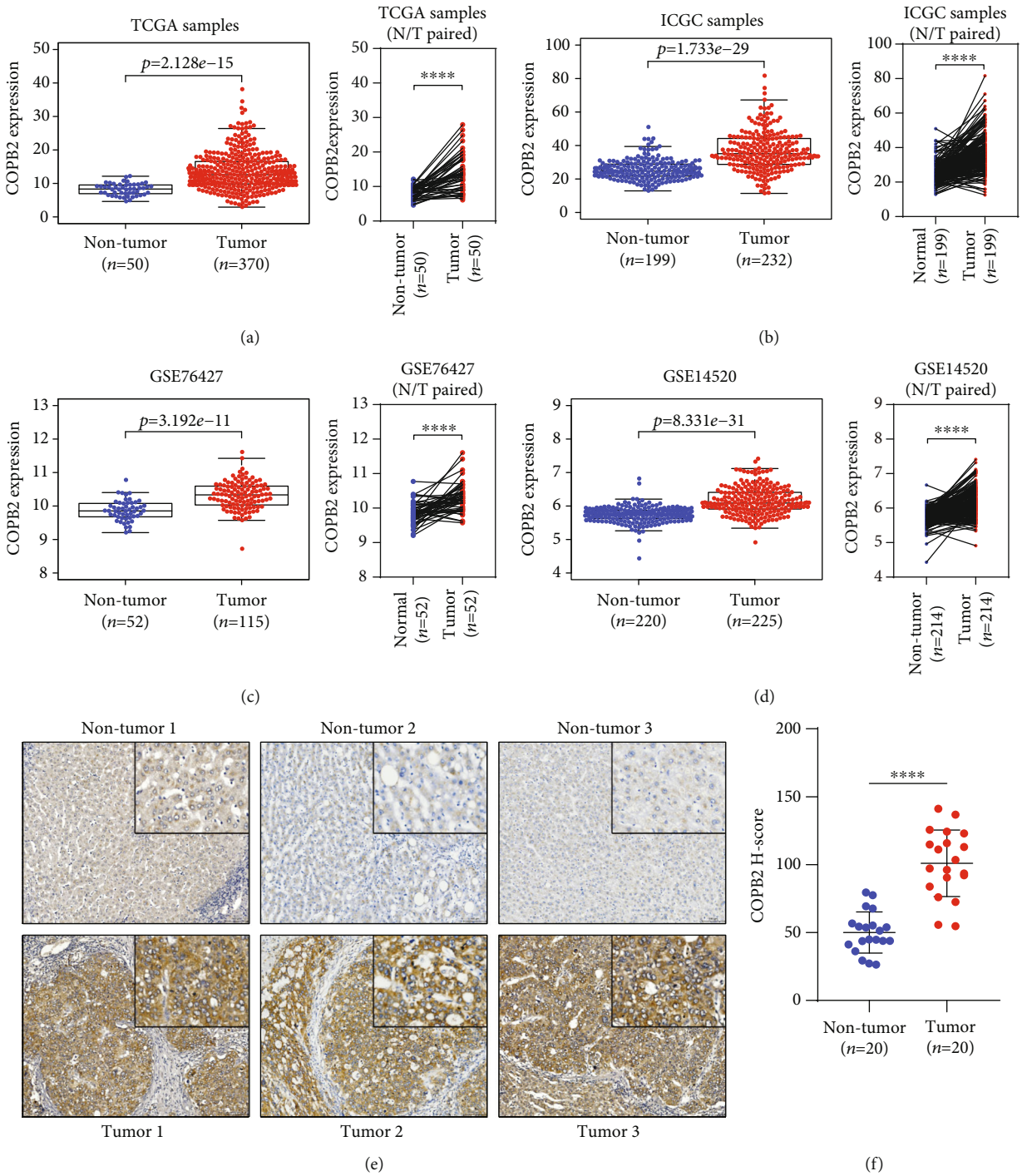


Figure 1: COPB2 overexpressed in HCC. (a) Comparison of COPB2 mRNA expression in tumor ($n = 370$) and nontumor tissues ($n = 50$) in patients with HCC from TCGA database using paired (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) and unpaired (Mann–Whitney U test, $p < 0.0001$) analyses. (b) Comparison of COPB2 mRNA expression in tumor ($n = 232$) and nontumor ($n = 199$) tissues in patients with HCC from ICGC database using paired (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) and unpaired (Mann–Whitney U test, $p < 0.0001$) analyses. (c) Comparison of COPB2 mRNA expression in tumor ($n = 115$) and nontumor tissues ($n = 52$) in patients with HCC from the GSE76472 dataset using paired (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) and unpaired (Mann–Whitney U test, $p < 0.0001$) analyses. (d) Comparison of COPB2 mRNA expression in tumor ($n = 225$) and nontumor tissues ($n = 220$) in patients with HCC from the GSE76472 dataset using paired (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) and unpaired (Mann–Whitney U test, $p < 0.0001$) analyses. (e, f) Immunohistochemical analysis of COPB2 in HCC tissues ($n = 20$) and adjacent nontumor tissues ($n = 20$) (t-test, $p < 0.0001$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

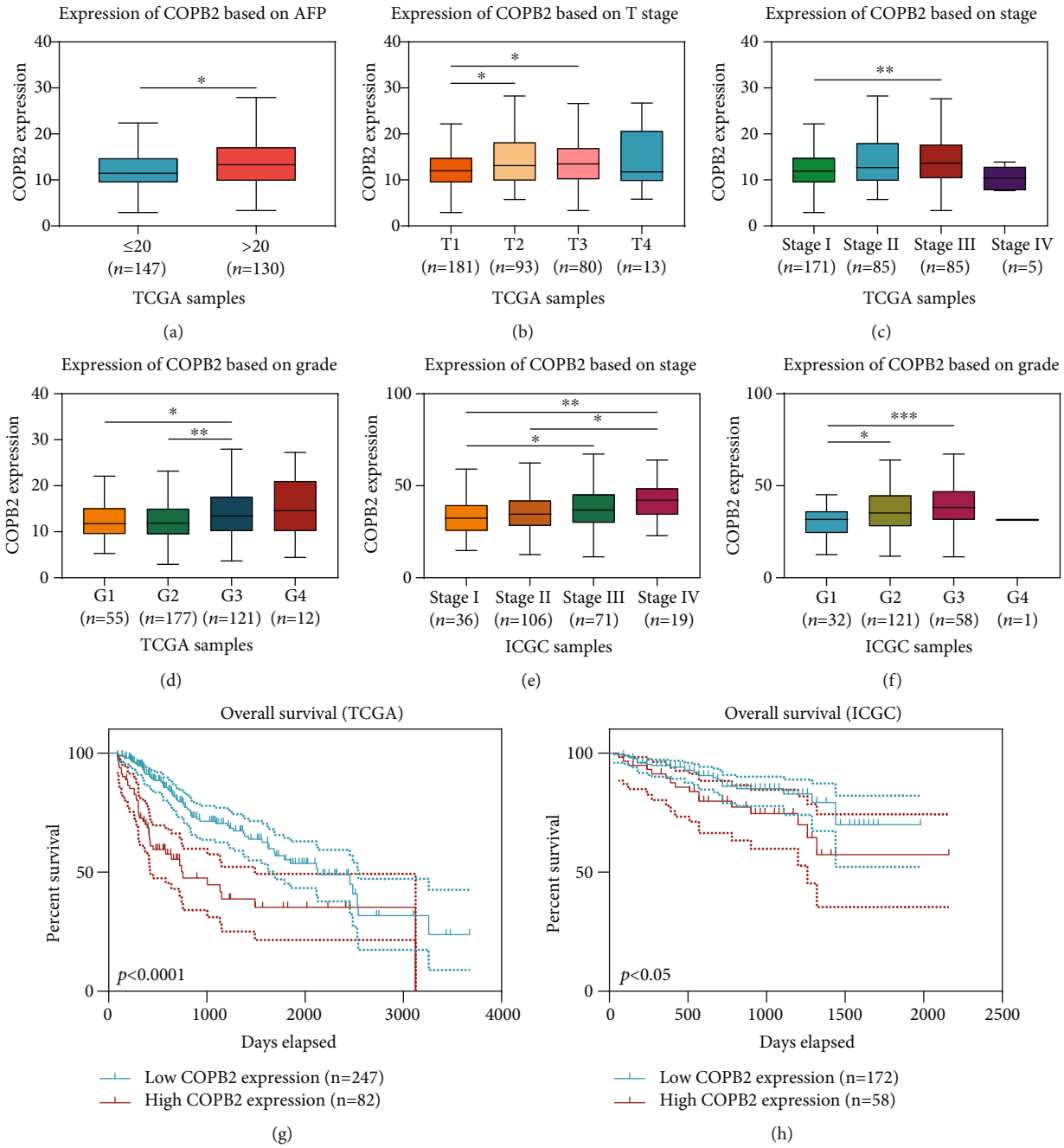


Figure 2: High COPB2 expression was correlated with poor prognosis in HCC patients. (a) Expression of COPB2 based on AFP in patients with HCC from TCGA cohort: AFP ≤ 20 ($n = 147$) vs. AFP > 20 ($n = 130$), $p < 0.05$ (Mann–Whitney U test). (b) Expression of COPB2 based on T stage in patients with HCC from TCGA cohort: T3 ($n = 80$) vs. T1 ($n = 181$), $p < 0.05$; T2 ($n = 93$) vs. T1 ($n = 181$), $p < 0.05$ (Mann–Whitney U test). (c) Expression of COPB2 based on stage in patients with HCC from TCGA cohort: Stage III ($n = 85$) vs. Stage I ($n = 171$), $p < 0.01$ (Mann–Whitney U test). (d) Expression of COPB2 based on grade in patients with HCC from TCGA cohort: G3 ($n = 121$) vs. G1 ($n = 55$), $p < 0.05$; G3 ($n = 121$) vs. G2 ($n = 177$), $p < 0.01$ (Mann–Whitney U test). (e) Expression of COPB2 based on stage in patients with HCC from the ICGC cohort: Stage IV ($n = 19$) vs. Stage I ($n = 36$), $p < 0.01$; Stage IV ($n = 19$) vs. Stage II ($n = 106$), $p < 0.05$; Stage III ($n = 71$) vs. Stage I ($n = 36$), $p < 0.05$ (Mann–Whitney U test). (f) Expression of COPB2 based on grade in patients with HCC from the ICGC cohort: G3 ($n = 58$) vs. G1 ($n = 32$), $p < 0.001$; G2 ($n = 121$) vs. G1 ($n = 32$), $p < 0.05$ (Mann–Whitney U test). (g, h) Effect of COPB2 expression level on overall survival of HCC patients in TCGA ($p < 0.0001$) and ICGC ($p < 0.05$) cohorts (cutoff: upper quartile) (Kaplan–Meier plot and log-rank test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

analysis of TCGA cohort also revealed similar results (Table 1).

Kaplan–Meier survival analysis (cases with a survival time of less than 30 days were not considered) indicated that HCC patients with high COPB2 expression had a more unfavorable prognosis than those with low COPB2 expression in both TCGA (Figure 2(g), high ($n = 82$) vs. low ($n = 247$), $p < 0.0001$) and ICGC cohorts (Figure 2(h), high ($n = 58$)

Table 1: Correlations between COPB2 mRNA expression and clinicopathological characteristics (logistic regression).

Clinical characteristics	Total (N)	OR in COPB2 expression	p value
Age (continuous)	370	0.986 (0.971–1.001)	0.067
Sex (male vs. female)	370	0.726 (0.468–1.122)	0.150
Status (with tumor vs. tumor-free)	343	1.154 (0.732–1.821)	0.538
Vascular invasion (positive vs. negative)	314	1.000 (0.627–1.594)	1.000
AFP (>20 vs. ≤20)	277	1.616 (1.006–2.606)	0.048*
T stage (T3+T2 vs. T1)	354	1.539 (1.013–2.345)	0.044*
Stage (III vs. I)	256	1.744 (1.033–2.969)	0.038*
Grade (G4+G3 vs. G2+G1)	365	1.746 (1.136–2.695)	0.011*

OR: odds ratio. *$p < 0.05$.

Table 2: Univariate and multivariate analyses of the correlation of COPB2 with OS among HCC patients from TCGA cohort.

Clinical characteristics	HR	p value
Univariate analysis		
Age (continuous)	1.250 (0.882–1.772)	0.210
Sex (male vs. female)	0.805 (0.564–1.147)	0.230
BMI (continuous)	0.974 (0.941–1.007)	0.124
T stage (T4/T3/T2/T1)	1.665 (1.390–1.993)	<0.0001****
Lymph nodes (positive vs. negative)	1.948 (0.477–7.952)	0.353
Distant metastasis (positive vs. negative)	3.820 (1.201–12.146)	0.023*
Stage (IV/III/II/I)	1.652 (1.349–2.024)	<0.0001****
Grade (G4/G3/G2/G1)	1.127 (0.892–1.424)	0.317
Tumor status (with tumor vs. tumor-free)	1.604 (1.116–2.306)	0.011*
Family cancer history (yes vs. no)	1.182 (0.819–1.707)	0.372
New tumor event (yes vs. no)	1.335 (0.932–1.913)	0.116
COPB2 expression (continuous)	1.068 (1.037–1.099)	<0.0001****
Multivariate analysis		
T stage (T4/T3/T2/T1)	2.074 (0.816–5.270)	0.125
Distant metastasis (positive vs. negative)	1.642 (0.282–9.544)	0.581
Stage (IV/III/II/I)	0.772 (0.269–2.215)	0.630
Tumor status (with tumor vs. tumor-free)	1.002 (0.487–2.064)	0.995
COPB2 expression (continuous)	2.011 (1.111–3.641)	0.021*

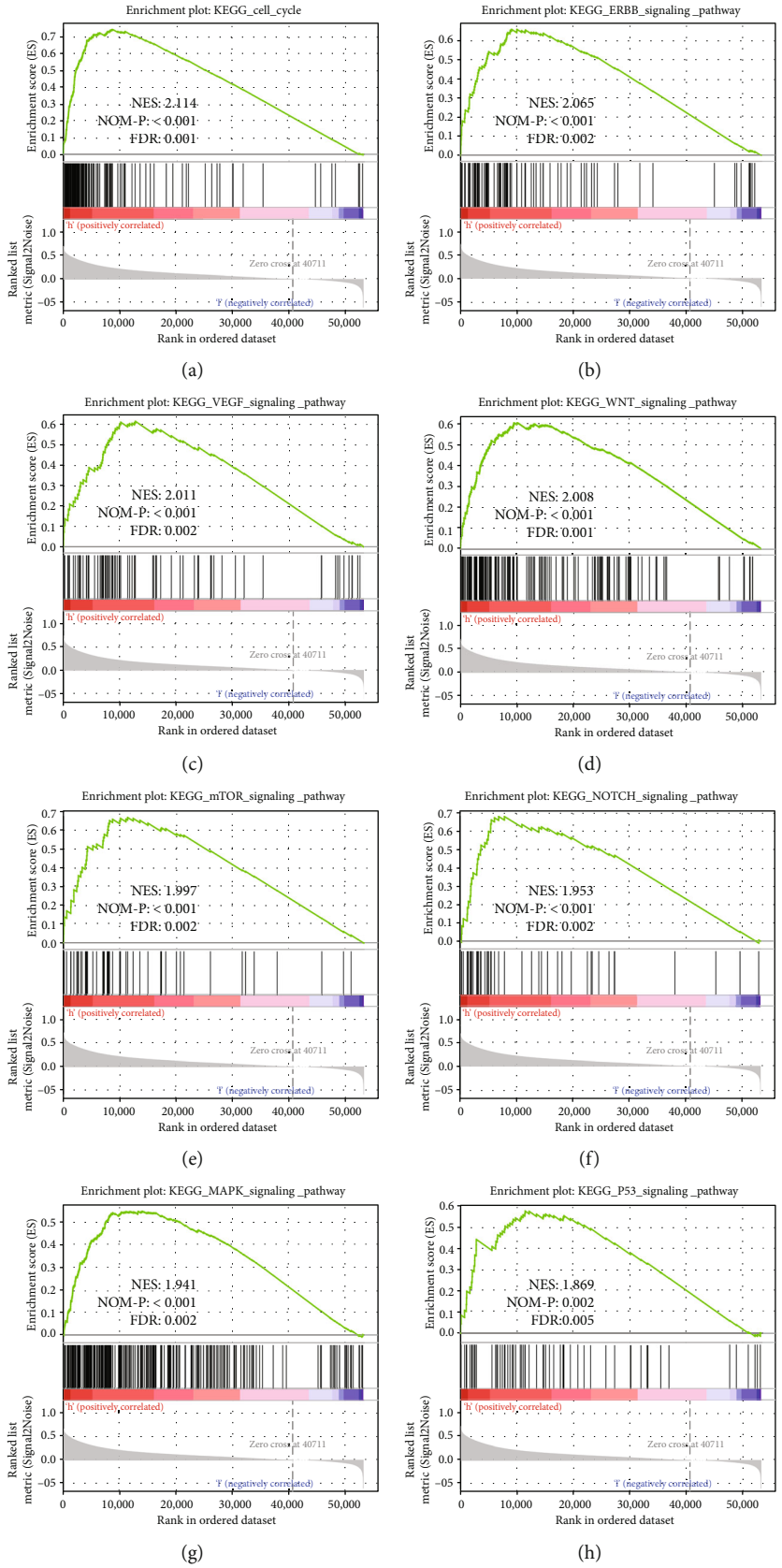
HR: hazard ratio. *$p < 0.05$ and ****$p < 0.0001$.

vs. low ($n = 172$), $p < 0.05$). The upper quartile value of COPB2 expression levels was used as the cutoff point [22].

Univariate and multivariate Cox proportional hazard regression analyses were performed on TCGA cohort. In the univariate Cox analysis, shorter overall survival (OS) was found in those with higher expression of COPB2 (hazard ratio (HR) = 1.068, 95% confidence interval (CI): 1.037–1.099, $p < 0.0001$), higher T stage (HR = 1.665, 95% CI: 1.390–1.993, $p < 0.0001$), worse pathological stage (HR = 1.652, 95% CI: 1.349–2.024, $p < 0.0001$), and "with tumor" status (HR = 1.604, 95% CI: 1.116–2.306, $p < 0.05$) (Table 2). However, in the multivariate Cox analysis, worse OS was only significantly associated with high expression of COPB2 (HR = 2.011, 95% CI: 1.111–3.641, $p < 0.05$) (Table 2). This indicates that COPB2 was an independent prognostic factor for HCC.

In summary, the above results indicated that high COPB2 expression correlated with poor prognosis in HCC.

3.3. GSEA Identified COPB2-Related Biological Signaling Pathways in HCC. To explore the biological signaling pathways involved in COPB2 expression in HCC, we performed GSEA of the high and low COPB2 expression groups in TCGA cohort. The results revealed a great number of significant differences (false discovery rate (FDR) < 0.05, $p < 0.05$) in the enrichment of the Molecular Signatures Database (MSigDB) Collection (c2.cp.kegg.v7.2.symbols.gmt), and we observed that the cell cycle (Figure 3(a), normalized enrichment score (NES): 2.114, FDR: 0.001, $p < 0.001$), ERBB signaling pathway (Figure 3(b), NES: 2.065, FDR: 0.002, $p < 0.001$), VEGF signaling pathway (Figure 3(c), NES:



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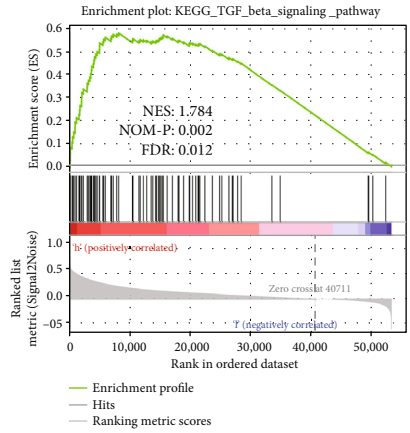


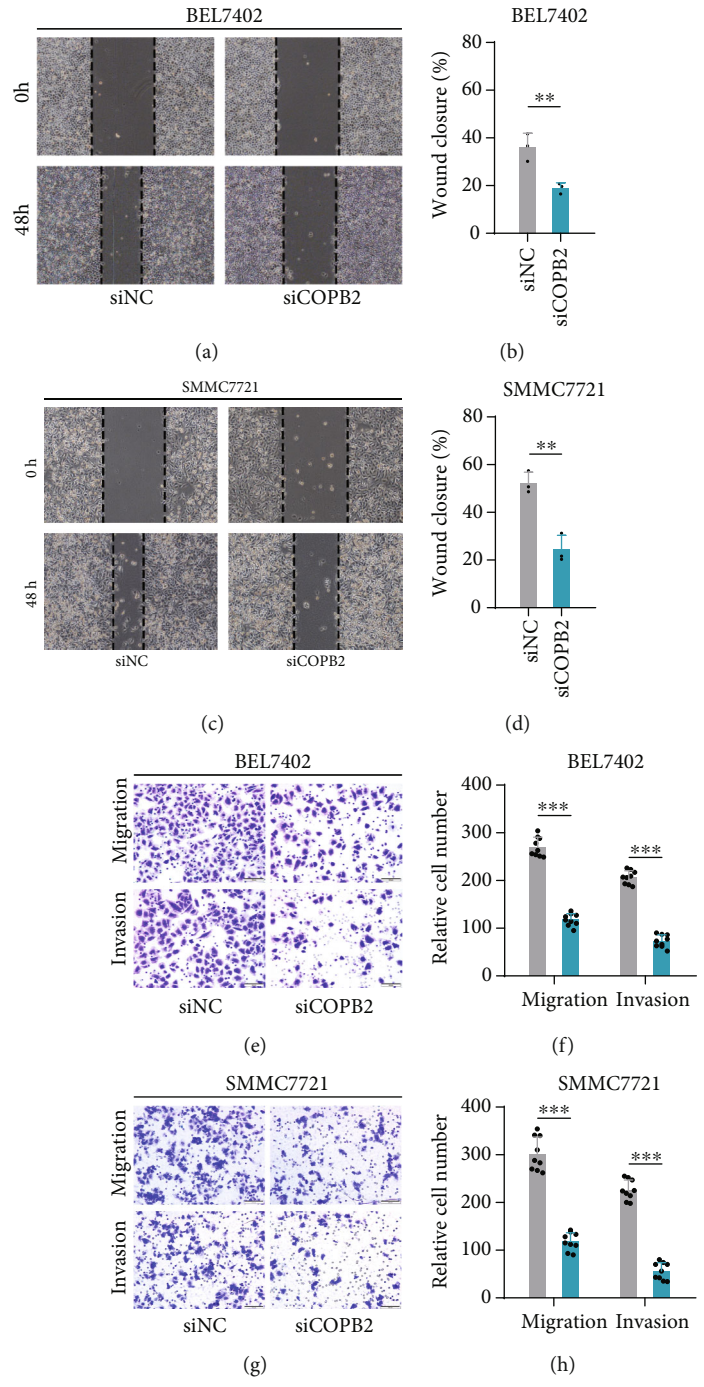
(i)

Figure 3: GSEA identified COPB2-related biological signaling pathways in HCC. (a) Cell cycle. (b) ERBB signaling pathway. (c) VRGF signaling pathway. (d) WNT signaling pathway. (e) mTOR signaling pathway. (f) NOTCH signaling pathway. (g) MAPK signaling pathway. (h) P53 signaling pathway. (i) TGF-β signaling pathway. ES: enrichment score; NES: normalized ES; NOM-P: normalized p value; FDR: false discovery rate.

2.011, FDR: 0.002, $p < 0.001$), WNT signaling pathway (Figure 3(d), NES: 2.008, FDR: 0.001, $p < 0.001$), mTOR signaling pathway (Figure 3(e), NES: 1.997, FDR: 0.002, $p < 0.001$), NOTCH signaling pathway (Figure 3(f), NES: 1.953, FDR: 0.002, $p < 0.001$), MAPK signaling pathway (Figure 3(g), NES: 1.941, FDR: 0.002, $p < 0.001$), P53 signaling pathway (Figure 3(h), NES: 1.869, FDR: 0.005, $p < 0.01$), and TGF-β signaling pathway (Figure 3(i), NES: 1.784, FDR: 0.012, $p < 0.01$) were differentially enriched in those with the high COPB2 mRNA expression phenotype. The results indicated that COPB2 may play a vital role in the occurrence and progression of HCC.

3.4. Knockdown of COPB2 Suppressed Migration and Invasion of HCC Cell Lines. GSEA results showed that overexpression of COPB2 positively correlated with the activation of many tumor-related pathways in HCC. To verify the results of GSEA, we performed a series of experiments at the cellular level. All experiments were repeated at least three times. Wound healing assays showed that the migration ability of the COPB2 knockdown group was significantly weaker than that of the vector-transfected control group in both the BEL7402 (Figures 4(a) and 4(b), $p < 0.01$) and SMMC7721 (Figures 4(c) and 4(d), $p < 0.01$) cell lines. Transwell assays verified that downregulation of COPB2 significantly inhibited the migration (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$) and invasion (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$) abilities of both cell lines (Figures 4(e)–4(h)). Moreover, we also measured the change in epithelial-mesenchymal transition- (EMT-) related protein expression levels using western blotting assays. The results revealed that the protein level of E-cadherin was markedly elevated (BEL7402, $p < 0.01$; SMMC7721, $p < 0.01$), while the expression of N-cadherin (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$), vimentin (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$), and Snail (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$) was significantly downregulated in both cell lines after COPB2 knockdown (Figures 4(i)–4(k)).

3.5. Silencing of COPB2 Inhibits the Proliferation by Inhibiting mTOR Signaling. In order to explore whether COPB2 can affect the proliferation of HCC, we performed CCK-8 assays. As expected, cells transfected with siCOPB2 had a lower rate of proliferation than siNC-treated cells in both BEL7402 (Figure 5(a); 24 h: $p < 0.01$, 48 h: $p < 0.0001$, 72 h: $p < 0.0001$) and SMMC7721 (Figure 5(b); 24 h: $p < 0.01$, 48 h: $p < 0.001$, 72 h: $p < 0.0001$) cell lines. In addition, we examined their cell cycle distribution and observed that compared with the siNC group, there was a significant increase in the number of cells in the G0/G1 phase and a decrease in the number of cells in the G2/M phase in the siCOPB2 group in both the BEL7402 (Figure 5(c), G0/G1: $p < 0.001$; G2/M: $p < 0.001$) and SMMC7721 (Figure 5(d), G0/G1: $p < 0.001$; G2/M: $p < 0.001$) cell lines. GSEA results suggested that the activation of the mTOR signaling pathway was closely associated with overexpression of COPB2 in HCC. To further confirm this, we performed immunohistochemical staining on tumor tissues and matched nontumor tissues in HCC patients. As expected, the results show that phospho-mTOR was significantly overexpressed in tumor tissues (Figures 5(e) and 5(f), $p < 0.0001$). In addition, the activity of this pathway of HCC cell lines was examined using a western blotting assay. In the present study, we observed that after knocking down COPB2, the expression level of mTOR (BEL7402, $p < 0.01$; SMMC7721, $p < 0.01$) and p70 S6K (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$) as well as phospho-mTOR (BEL7402, $p < 0.01$; SMMC7721, $p < 0.01$), phospho-p70 S6K (BEL7402, $p < 0.01$; SMMC7721, $p < 0.01$), and their downstream protein cyclin D1 (BEL7402, $p < 0.001$; SMMC7721, $p < 0.001$) decreased in both cell lines (Figures 5(g)–5(i)). These results suggest that silencing of COPB2 inhibits cell proliferation and that the mTOR signaling pathway plays an important role.



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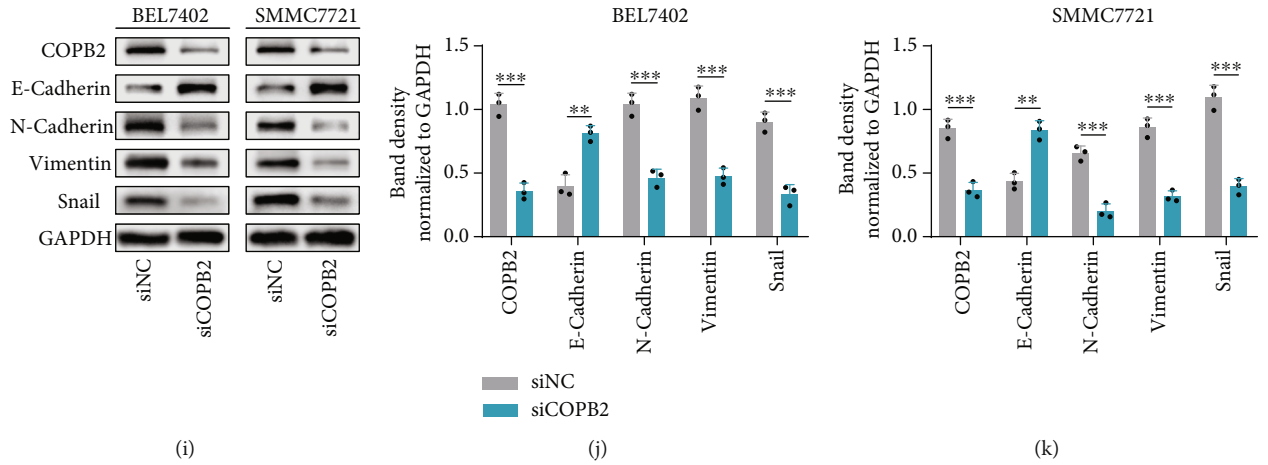


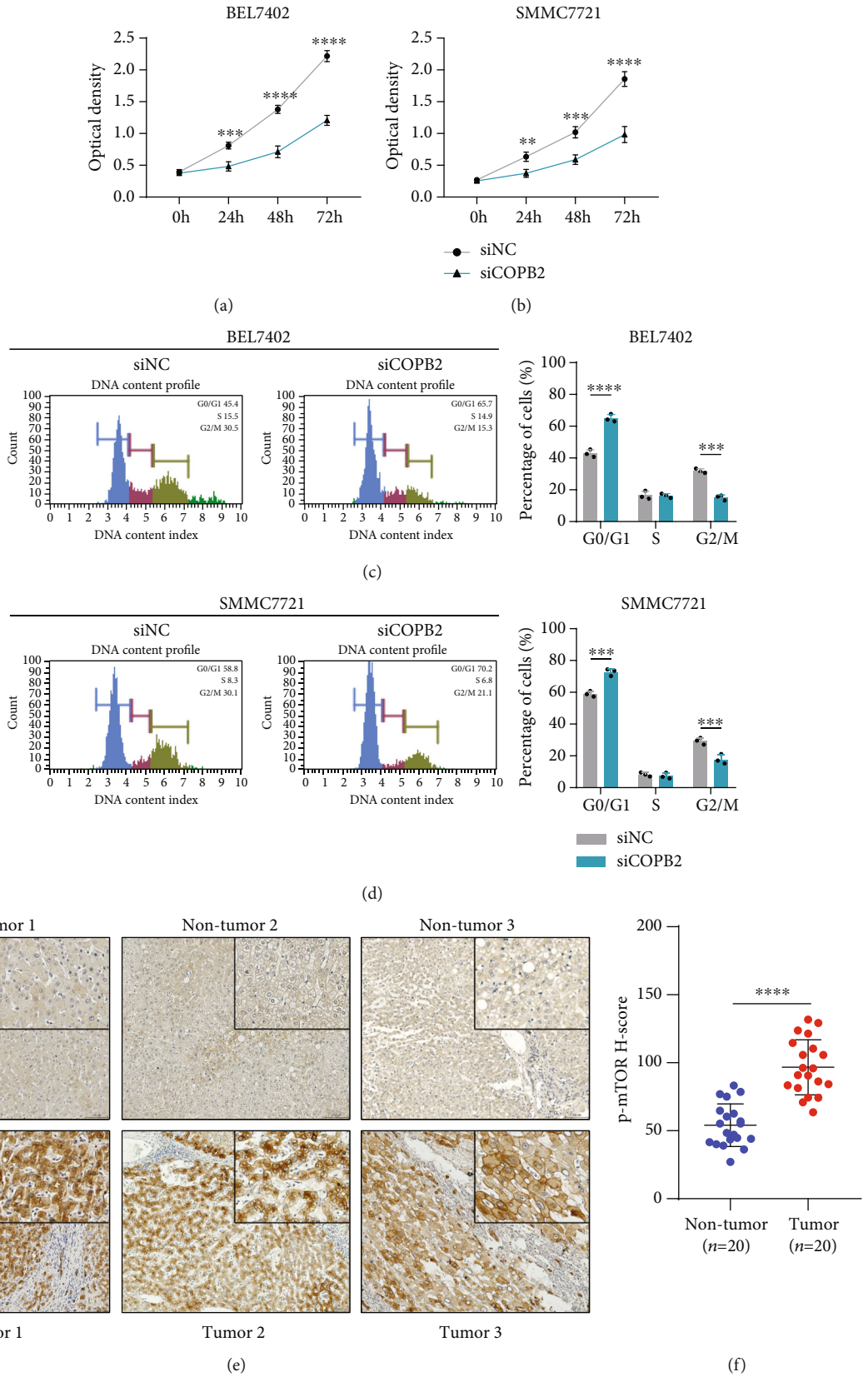
Figure 4: Knockdown of COPB2 suppressed migration and invasion in HCC cell lines. (a, c) Wound healing assays detected the cell migration ability of BEL7402 and SMMC7721 cells transfected with siNC or siCOPB2; the representative images were obtained at different time points. (b, d) Statistical analysis of the results of wound healing assays ($n = 3$). (e, g) Transwell assays were used to detect the cell migration and invasion ability of BEL7402 and SMMC7721 cells transfected with siNC and siCOPB2; the representative images are displayed. (f, h) Statistical analysis of the results of the Transwell assays ($n = 9$). (i) Representative images of western blotting analysis of COPB2, E-cadherin, N-cadherin, vimentin, Snail, and GAPDH in BEL7402 and SMMC7721 cells transfected with siNC and siCOPB2. GAPDH was used as the loading control. (j, k) Statistical analysis of gray values of western blotting assays ($n = 3$). All data were analyzed using the t-test and are displayed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

4. Discussion

HCC accounts for approximately 80% of primary liver cancers [1]. Due to its asymptomatic disease progression and lack of effective methods to make an early diagnosis, HCC is often diagnosed at an advanced stage [6]; its typically late-stage presentation, limited treatment options, and aggressive nature lead to it having a very poor prognosis [4, 24, 25]. In China, digestive tract cancers account for 36.4% of cancer-related deaths, of which liver cancer account for more than one-third [2]. Therefore, there is an urgent need to identify effective biomarkers for the diagnosis and prognosis of HCC, as well as therapeutic targets.

COPB2 is a 102 kDa protein that was first identified in 1993 [26, 27]. Previous research confirmed that COPB2 is an element of non-clathrin-coated vesicles and is involved in regulating membrane transport in extracellular pathways [9, 28]. In addition, as a subunit of the Golgi coatomer complex, COPB2 is essential for retrograde transport from the Golgi complex to the endoplasmic reticulum [11–13]. Compared with normal cells, the biosynthetic activity of tumor cells is abnormally vigorous [29]. As is well known, the Golgi complex plays an important role in anabolism; thus, COPB2 is certain to play a very important role in the occurrence and progression of tumors. Recently, the functions of COPB2 in tumors have been increasingly studied. In gliomas, COPB2 has been reported to be an important factor in the regulation of the immune microenvironment, and its high expression is related to adverse outcomes [14]. In breast cancer, COPB2 may predict metastasis [15]. In gastric cancer, COPB2 can affect the growth and apoptosis of gastric cancer cell lines via the RTK signaling pathway [30]. In lung adenocarcinoma, COPB2 was confirmed to be overexpressed and negatively correlated with survival, and COPB2 downregulation enhanced apoptosis and repressed proliferation and tumorigenesis in lung adenocarcinoma cells [31]. In prostate cancer, COPB2 has also been shown to be highly expressed and can promote PC-3 cell proliferation and inhibit apoptosis by affecting its cell cycle [16]. Downregulation of COPB2 could inhibit the growth of human cholangiocellular carcinoma cells [32]. It has also been reported that reduced editing of *COPA*, an important paralog of COPB2, has been implicated in the pathogenesis of HCC, and editing of $COPA^{WT}$ may switch it from a tumor-promoting gene to a tumor suppressor by deactivating the PI3K/AKT/mTOR pathway through downregulation of caveolin-1 (CAV1) [20]. These findings suggest an essential role of COPB2 in the occurrence and progression of tumors, which provides a good theoretical basis for our study of the role of COPB2 in HCC.

With the advancement of technology, high-throughput sequencing technology has been increasingly used in cancer research [33, 34]. In the present study, we explored the role of COPB2 in human HCC and the underlying mechanism using database analysis combined with basic experiments. Bioinformatic analysis based on TCGA, ICGC, and GEO databases revealed that COPB2 mRNA levels were higher in HCC tissues than in nontumor tissues. At the same time, we confirmed the high expression of COPB2 protein in HCC tissues using immunohistochemical assay. The mRNA expression data and clinical information of HCC were then analyzed. Correlation and survival analyses showed that high COPB2 expression was closely correlated with advanced clinicopathological parameters (higher AFP, worse T stage, poor pathological stage, and higher grade) and worse prognosis. Univariate and multivariate Cox analyses indicated that COPB2 was an independent prognostic factor for HCC. GSEA suggested that various signaling pathways closely related to tumor occurrence and development [35–39] (e.g.,



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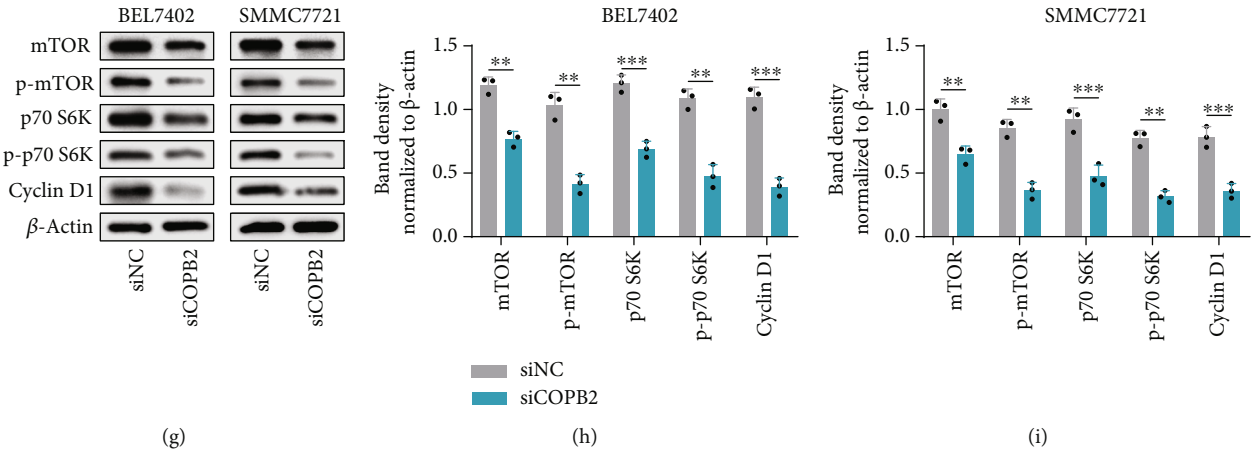

Figure 5: Silencing of COPB2 inhibits the proliferation by inhibiting mTOR signaling. (a, b) BEL7402 and SMMC7721 cells were transfected with siNC and siCOPB2, respectively, and cell viability was analyzed using the CCK-8 assays ($n = 5$) (two-way repeated measurement ANOVA with Sidak's multiple comparisons test). (c, d) Detection of the cell cycle distribution of BEL7402 and SMMC7721 cells after being transfected with siNC and siCOPB2 ($n = 3$) (t-test). (e, f) Immunohistochemical analysis of p-mTOR in HCC tissues ($n = 20$) and adjacent nontumor tissues ($n = 20$) (t-test). (g) Representative images of western blotting analysis of mTOR, p-mTOR, p70 S6K, p-p70 S6K, cyclin D1, and β-actin in BEL7402 and SMMC7721 cells transfected with siNC and siCOPB2. #x03B2;-actin was used as the loading control. (h, i) Statistical analysis of gray values of western blotting assays ($n = 3$) (t-test). All data are displayed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

mTOR signaling pathway, WNT signaling pathway, VEGF signaling pathway, and NOTCH signaling pathway) were differentially enriched in those with the high COPB2 expression phenotype. To further explore the role of COPB2 in HCC, we performed a series of experiments. Functional investigations indicated that downregulation of COPB2 significantly inhibited the proliferation, migration, and invasion capacity of HCC in vitro. In addition, mechanistic experiments demonstrated that deletion of COPB2 significantly restrained EMT and activation of the mTOR signaling pathway.

The occurrence and progression of tumors are associated with abnormal regulation of multiple signaling pathways. EMT plays a vital role in tumorigenesis and tumor progression and is closely related to tumor invasion and migration abilities [40, 41]. Common signaling pathways, such as the WNT, NOTCH, MAPK, and TGF-β signaling pathways, can activate EMT regulators [39, 42–44]. Evidence indicates that the mTOR signaling pathway governs cell growth and is activated in cancer [35, 45]. The GSEA results showed that these signaling pathways were all enriched in the high COPB2 expression group in HCC, and the results of function and mechanism experiments are also consistent with this.

The results of the current study showed that COPB2 is overexpressed in HCC tissues, associated with HCC prognosis, and plays a crucial role in the proliferation, migration, and invasion of HCC cell lines in vitro, indicating that COPB2 is a novel prognostic biomarker and promising therapeutic target for HCC.

This study has some limitations. First, the tumor tissue specimens of the patients were usually obtained during surgery; however, patients with distant metastasis generally do not have indications for surgery. Consequently, the expression data from this patient population are rarely obtained. Second, according to the results of the GSEA, COPB2 may also influence the progression of HCC through other signaling pathways other than the mTOR signaling pathway; however, the current research on the relationship between COPB2 and HCC is in its infancy, and a lot of work is needed to explore whether COPB2 can affect HCC through other pathways in subsequent studies. Finally, this study only included cases from two cohorts, and a multicenter study should be conducted in the future.

5. Conclusion

COPB2 is a novel prognostic biomarker and a promising therapeutic target of HCC.

Abbreviations

HCC: Hepatocellular carcinoma
COPB2: Coatomer protein complex subunit beta 2
TCGA: The Cancer Genome Atlas
ICGC: International Cancer Genome Consortium
GEO: Gene Expression Omnibus
GSEA: Gene set enrichment analysis
AFP: Alpha fetoprotein
COPI: Coatomer complex I
COPs: Coat proteins
COPA: Coatomer protein complex subunit alpha
OS: Overall survival
ES: Enrichment score
NES: Normalized enrichment score
CCK-8: Cell counting kit-8
EMT: Epithelial-mesenchymal transition.

Data Availability

The data used to support this study are available from the corresponding author upon request.

Ethical Approval

The present study was conducted according to the principles stated in the Declaration of Helsinki and approved by the Ethics Committee of Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University (No. 2019–032).

Consent

According to the guidelines of the ethics committee, written informed consent was obtained from all patients. All datasets were retrieved from public literature, and all written informed consent was obtained.

Conflicts of Interest

The authors report no conflicts of interest in this work.

Authors' Contributions

Jun Liu and Jiayao Zhang designed the research methods, performed the experiments, and analyzed the data. Xiaoyu Wang participated in experiments and data collection. Guangbing Li and Jingyi He collected tumor tissues. Ziwen Lu, Yang Yang, Yong Jiang, Liyong Jiang, and Feiyu Li revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

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Supplementary Materials

Table S1: the HCC patients' basic information of TCGA cohort. Table S2: the HCC patients' basic information of ICGC cohort. Table S3: basic characteristics of six HCC cohorts from GEO database. Figure S1: COPB2 expression analysis of four HCC cohorts from GEO database. *(Supplementary Materials)*

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Research Article

Intratumoral STING Agonist Injection Combined with Irreversible Electroporation Delays Tumor Growth in a Model of Hepatocarcinoma

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Background/Aim. Irreversible electroporation (IRE) showed promising results for small-size tumors and very early cancers. However, further development is needed to evolve this procedure into a more efficient ablation technique for long-term control of tumor growth. In this work, we show that it is possible to increase the antitumor efficiency of IRE by simmultaneously injecting c-di-GMP, a STING agonist, intratumorally. *Materials and Methods*. Intratumoral administration of c-di-GMP simultaneously to IRE was evaluated in murine models of melanona (B16.OVA) and hepatocellular carcinoma (PM299L). *Results*. The combined therapy increased the number of tumor-infiltrating IFN-γ/TNF-α-producing CD4 and CD8 T cells and delayed tumor growth, as compared to the effect observed in groups treated with c-di-GMP or IRE alone. *Conclusion*. These results can lead to the development of a new therapeutic strategy for the treatment of cancer patients refractory to other therapies.

1. Introduction

Irreversible electroporation (IRE) is an emerging alternative to multimodal ablative therapies for the liver [1], prostate [2], kidney [3], pancreas [4–6], or lung cancers [7]. The main use of IRE is aimed at the ablation of tumors that are in contact with vital vascular or nervous structures which must be preserved. Electroporation destroys tumor cells but it does not affect collagen-containing structures like vessels and nerves [8–10]. The advantages of IRE compared to other techniques are as follows: (i) the selectivity of the tissue affected [10]; (ii) the ability to specifically define the margins affected by the procedure [11]; (iii) the short time the treatment lasts; and (iv) the possibility of monitoring the effect of electroporation in real time [11]. All this makes IRE a therapeutic alternative in patients with tumors located in areas not surgically resectable near to vital structures.

Clinical trials showed safety and absence of serious adverse effects when IRE was used; however, its therapeutic efficacy remained poor [5, 12, 13]. It was suggested that there are islands of viable tumor cells remaining within ablated regions after IRE treatment, which may contribute to tumor development [14]. Lack of long-term efficacy of this technique might also be due to its limited capacity to induce an inflammatory reaction that favors the activation of an antitumor immune response. This is because IRE causes tumor cell death by apoptosis and not necrosis as in other techniques based on thermal ablation or radiation [15]. In previous work, we found that it was possible to improve the antitumor effect of IRE when combining it with the intratumoral injec-

tion of Poly-ICLC (Hiltonol) immediately before the IRE procedure [16]. Poly-ICLC is a synthetic analog that mimics double-stranded viral RNA, a ligand of pattern recognition receptors (PRR) including TLR3, MDA5, RIG-1, or the NLRP3 inflammasome that sense danger signals [17]. In addition to RNAs, double-stranded DNAs (dsDNA) are potent inducers of type I interferons (IFNs). There are a number of sensors of cytosolic dsDNA which can trigger different signaling pathways through the endoplasmic reticulum membrane protein STING (stimulator of IFN genes) [18] [19]. Indeed, in the presence of cytosolic double-stranded DNA (dsDNA), activated cyclic GMP-AMP synthase (cGAS) uses cytosolic ATP and GTP as substrates to catalyze the production of cyclic dinucleotides (CDNs) (reviewed in [20]). Upon binding to CDNs, STING translocates from the ER to the Golgi apparatus and further to the perinuclear microsomes and activate TBK-1/IRF-3 and NF-κB signaling pathways inducing robust type I IFNs and pro-inflammatory cytokines, which can trigger adaptive immune responses against tumors [21, 22]. A number of natural and synthetic STING agonists are being tested in preclinical models and in the clinic for the immunotherapy of cancer. However, these molecules are susceptible to enzymatic degradation, having low bioavailability in target tissues and producing unwanted toxicities. New drug delivery systems are being explored to address these challenges [23].

Our main goal in the present work was to evaluate the effectiveness of IRE concomitant to the administration of a STING agonist to improve mice survival after a long-term follow-up. We have made the proof of concept in murine models of melanoma and hepatocarcinoma.

2. Materials and Methods

2.1. Cell Lines and Mice. B16-OVA (ATCC, American Type Culture Collection) and PM-299L (provided by Dr. Lujambio, NY) cell lines were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (CM medium). Specific pathogen-free, 7-10-week-old female C57BL/6 wild-type mice (Charles River) were used in agreement with the ethical directives of the Spanish veterinary authorities. They were housed in appropriate animal care facilities during the experiments and handled following the international guidelines required for experimentation with animals. Institutional ethical committee approved the experiments (Ref. 111-15).

2.2. In Vivo Experiments: Ire Treatment and Tumor Follow-Up. B16.OVA melanoma cells or PM299L HCC cells were injected ($5x10^5$ cells/mouse), subcutaneously (s.c.) in C57BL/6 mice ($n = 5 - 8$) purchased by Harlan (Barcelona, Spain). Ten days after tumor cell injection, when the tumors grew to 5 mm in diameter, mice were randomly distributed into different experimental groups.

Irreversible electroporation was carried out using the ECM 830 Square Wave Electroporation System, using specific tweezers (edges of 2 mm) for the fixation of the tumor for the IRE treatment. IRE consisted in twenty consecutive pulses of 2500 V/cm (0.1 msec each) with 0.5 s intervals between pulses. When indicated, 25 μL of a solution containing 1 mg/mL c-di-GMP STING agonist (InvivoGen) was injected intratumorally into the space defined by the tweezers. In an experimental group, c-di-GMP administration was done immediately before electroporation (IRE + c-di-GMP group). In another experimental group (c-di-GMP group), c-di-GMP was administered intratumorally exactly as described above, but without the administration of the electroporation current. IRE group received only the electroporation treatment alone without the c-di-GMP administration. Tumor size, represented as the multiplication of two perpendicular diameters (mm^2), was measured at different time points. According to the institutional guidelines, mice were sacrificed if the mean tumor diameter was greater than 20 mm^2.

2.3. Flow Cytometry. For characterization experiments, PM299L tumor-bearing mice were treated as indicated, and 10 days later, mice were sacrificed to analyze immune infiltrate by flow cytometry. Tumors were excised and digested with collagenase D (400 U/mL) and DNase-I (50 μg/mL, Roche) for 20 min at 37°C. The spleens were mashed in PBS. Red blood cells were lysed by ACK buffer (Sigma). For functional analyses, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL) in the presence of GolgiStop and GolgiPlug (BD Biosciences). After 5 hours, cells were incubated with Zombie NIR Fixable dye (BioLegend) and stained with fluorochrome-labeled mAbs against CD45.2 (104), CD8 (XMG1.4), CD4 (RMA4-5), and CD44 (IM7) in the presence of purified anti-CD16/32 mAb. For intracellular staining, cells were treated with the BD Fixation/Perm buffer (BD Biosciences) and stained with anti-IFN-γ (XMG1.2) and anti-TNFα (MP6-XT22) mAbs. Samples were acquired on a FACSCanto-II cytometer (BD Biosciences). Data were analyzed using the FlowJo software (TreeStar).

2.4. Statistical Analysis. Normality was assessed with the Shapiro-Wilk W test. Statistical analyses were performed using parametric (Student's t test and one-way ANOVA with Tukey's multiple comparison) and nonparametric (Mann–Whitney U and Kruskal-Wallis) tests. GraphPad Prism for Windows was used for statistical analysis. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Irreversible Electroporation (IRE) in Combination with Intratumor Administration of c-di-GMP Adjuvant Has Therapeutic Effect in a Murine Model of Melanoma. IRE produces cellular destruction and the release of tumor-specific antigens, which might be captured by antigen presenting cells to initiate the induction of an antitumor immune responses. However, the tumor microenvironment is not favorable for antitumor immune priming. We proposed that utilizing an immunotherapeutic approach in combination with IRE might favor the induction of stronger antitumor immune responses. In order to do this, IRE was combined with the simultaneous injection of the immunostimulatory agent and STING agonist,

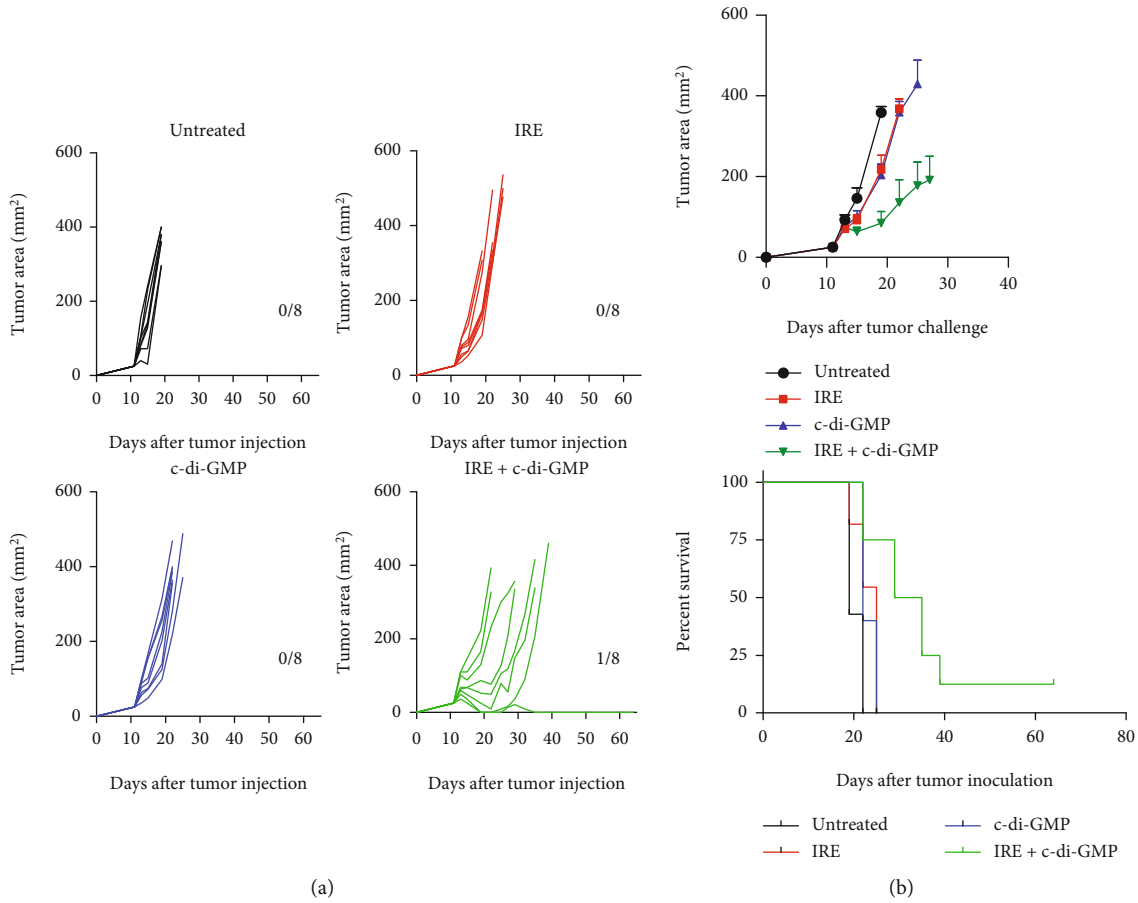

Figure 1: Treatment of B16.OVA tumor cells by irreversible electroporation plus c-di-GMP. Mice were challenged s.c. with B16-OVA tumor cells and at days 7-10, when tumors reached 5 mm in diameter, they were treated i.t. as indicated. (a) Each curve represents tumor mean diameter for an individual mouse. Numbers of mice free of tumors out of the total animals per group are indicated. (b) The Kaplan-Meier plots of the percentage of mice survival are represented. Log-rank (Mantel-Cox) test. $p < 0.05$.

c-di-GMP. To evaluate this combination therapy, we first used a murine model of melanoma based on the administration of B16.OVA tumor cells. Mice bearing B16.OVA were treated with (i) IRE, (ii) intratumoral injection of c-di-GMP, (iii) intratumoral injection of c-di-GMP immediately accompanied by IRE, or (iv) left untreated (control group).

IRE treatment or c-di-GMP treatment alone did not show any effect on tumor kinetics and did not significantly decreased tumor growth compared to the untreated group (Figures 1(a) and 1(b)). However, mice that received IRE + c-di-GMP combination treatment showed a significant delay in tumor growth, resulting in 1 out of 8 mice completely rejecting the tumor (Figure 1(a)). Survival was significantly improved in those mice compared to single treatment groups and the untreated control group ($p < 0.05$; Figure 1(b)).

3.2. IRE in Combination with c-di-GMP Has a Therapeutic Effect in a Murine Model for Hepatocellular Carcinoma. IRE is an emerging alternative to ablative therapies for liver cancer [1]. Even if results were particularly promising for small-size and very early-stage hepatocellular carcinomas (HCC), tumor recurrence is still high [24, 25]. We tested if combination of IRE with the c-di-GMP adjuvant could improve the efficacy of IRE in a murine model of HCC. C57BL/6 mice were injected with PM-299L hepatoma cells subcutaneously. Seven days later, mice were treated with (i) IRE, (ii) intratumoral injection of c-di-GMP, or (iii) intratumoral injection of c-di-GMP followed immediately by IRE or (iv) left untreated. It was observed that IRE treatment alone or c-di-GMP alone cured 16.6% and 20% of mice, respectively (Figure 2(a)). Surprisingly, 66.7% of mice responded to IRE + c-di-GMP combination therapy (Figure 2(a)) with 4 out of 6 mice totally rejecting established tumors. On the other hand, only 1 out of 5 or 1 out of 6 mice were cured after c-di-GMP or IRE monotherapies, respectively ($p < 0.05$; Figure 2(b)). We repeated the experiments but using male mice and the same treatments schedules. Combination therapy c-di-GMP plus IRE was also able to significantly delay tumor growth and mice survival (Figures 2(d)–2(f)), although the effect was less pronounced than that found in female mice. No effect was observed when mice were treated with monotherapies.

In order to evaluate the antitumor immune response in vivo, we repeated the experiment with the same treatment options but sacrificing the mice ten days after tumor injection. The phenotype and functionality of tumor infiltrates was then analyzed. Tumor size at the day of sacrifice was significantly lower in mice treated with the combination therapy (both measured as tumor diameter and as tumor weight,

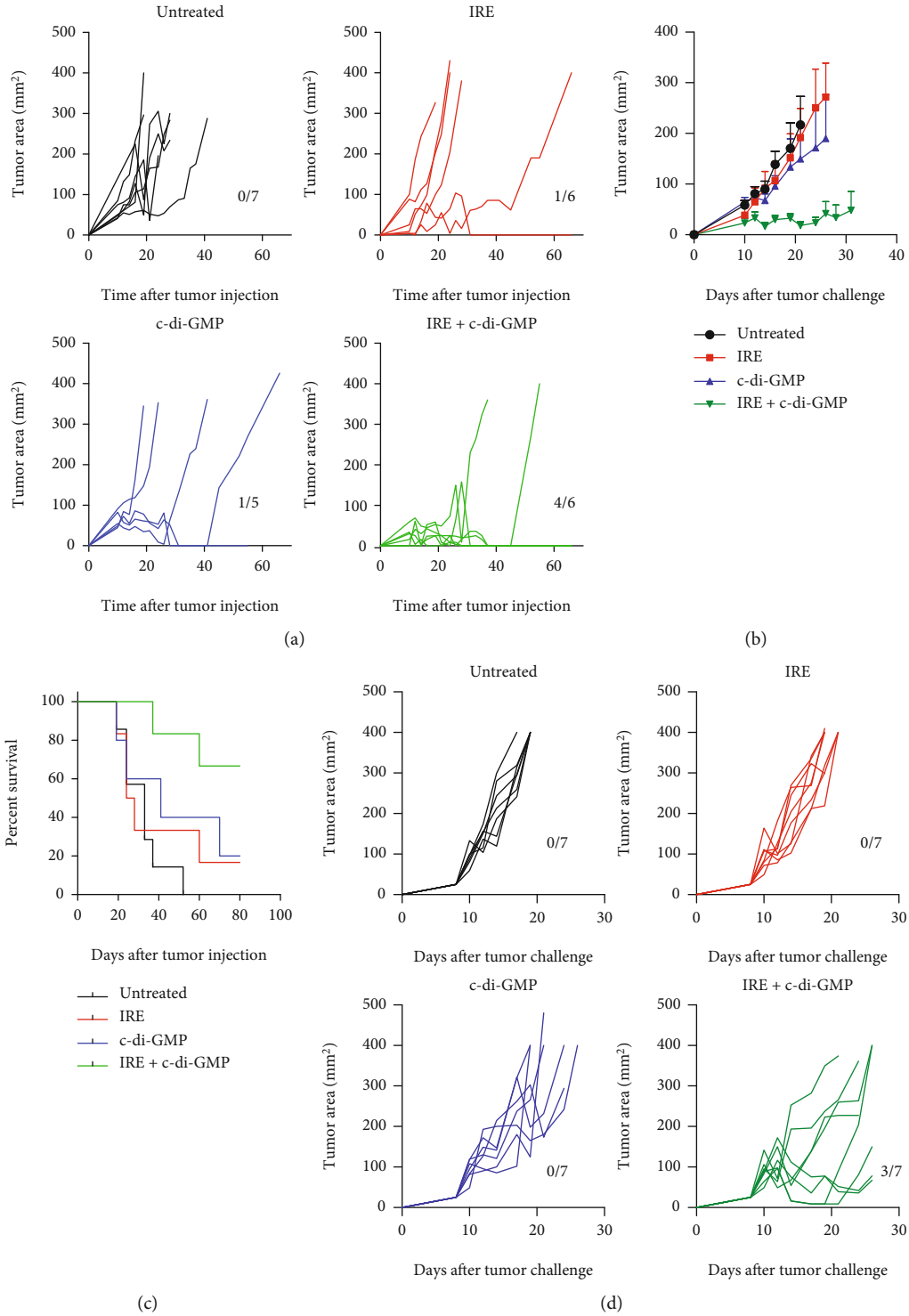

Figure 2: Continued.

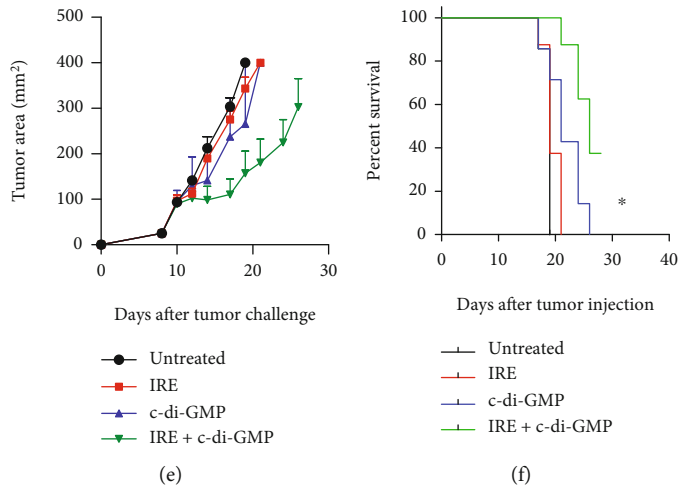

Figure 2: Treatment of PM299L tumor cells by irreversible electroporation plus c-di-GMP. Mice were challenged s.c. with PM299L tumor cells and at days 7-10, when tumors reached 5 mm in diameter, they were treated i.t. as indicated. (a) Each curve represents tumor mean diameter for an individual mouse. Numbers of mice free of tumors out of the total animals per group are indicated. (b) The Kaplan-Meier plots of the percentage of mice of survival are represented. Log-rank (Mantel-Cox) test, $p < 0.05$.

Figure 3(a)). Flow cytometric analysis of tumor-infiltrating lymphocytes showed a significant increase in the number of leukocytes (percentage of $CD45^+$ cells/mg of tumor) in mice treated with c-di-GMP alone or with c-di-GMP combined with IRE (Figure 3(b)). These differences were also observed in the percentage of activated $CD4^+$ and $CD8^+$ infiltrating lymphocytes (the percentage of $CD44^{high}CD4^+$ and $CD44^{high}CD8^+$ T cells) (Figure 3(c) and 3(d)). Importantly, these two groups showed a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells that simultaneously expressed TNF-α and IFN-γ, and in the percentage of IFN-γ-producing NK cells (Figures 3(e)–3(g) and Figure S1). These results suggest that intratumoral administration of c-di-GMP induced a proinflammatory microenvironment favorable for T cell/NK cell activation. IRE treatment alone did not induce tumor infiltration of immune cells. Interestingly, the combined therapy of c-di-GMP and IRE was able to significantly increase the percentage of infiltrating activated IFN-γ and TNF-α-producing $CD8^+$ T cells, suggesting that this combination therapy favors the activation of an antitumor immune response able to control tumor growth more efficiently.

4. Discussion

IRE is a promising, low-invasive technique for the ablation of solid tumors. Unlike thermal ablation techniques, IRE treatment does not damage the surrounding extracellular matrix, vessels, nerves, and neighboring normal tissue [12, 13, 26, 27]. Clinical trials have shown safety and absence of serious adverse effects related to the procedure. However, the therapeutic efficacy is poor [5, 12, 13], and high incidence of short-term recurrences was reported [12, 28, 29]. Some studies suggest that the remaining islands of viable tumor cells within ablated regions after IRE treatment are responsible for higher resistance to pore formation [14]. It is probable that these remaining IRE resistant cells may continue tumor development and reduce the therapeutic efficacy of this technique.

Long-term tumor growth control can be achieved by eliciting a strong antitumor immune response. However, IRE alone does not induce favorable inflammatory conditions to facilitate antitumor T cell priming. As shown in this work, IRE treatment did not augment T cell infiltration of the tumor or improve infiltrating T cell activation state. IRE-induced cellular destruction may lead to the release of a substantial amount of tumor-specific antigens that can be engulfed by dendritic cells (DC, the professional antigen presenting cells) for their presentation to tumor-specific T lymphocytes. However, T lymphocyte activation is only achieved if DCs are in a mature stage. This maturation process is highly impaired by the immunosuppressive tumor microenvironment. Modifying the tumor microenvironment by introducing molecules that promote the maturation of dendritic cells might favor the activation of an antitumor immune response. We speculated that intratumoral injection of factors with proinflammatory properties, like c-di-GMP, might synergize with IRE technique to elicit antitumor T cell responses.

In previous work, we showed that the therapeutic effect of IRE can be improved when combined with simultaneous intratumoral administration of Poly-ICLC, a TLR3 agonist that mimics a viral infection and activates a strong innate immunity [16]. In addition to TLR ligands, the cGAS–STING axis was identified as an important regulator of immunity by mediating type I IFN production in response to cytosolic DNA [30, 31]. Type I IFN production elicited through the STING pathway has an essential role in the development of antitumor immunity by facilitating antigen cross-presentation by DCs (reviewed in [32]). DNA sensing by STING triggers the production of type I IFN by DCs and facilitates effective cross-priming of tumor-specific $CD8^+$ T cells [33]. The proinflammatory potential of STING signaling has prompted many laboratories towards the search and development of small molecule modulators targeting the

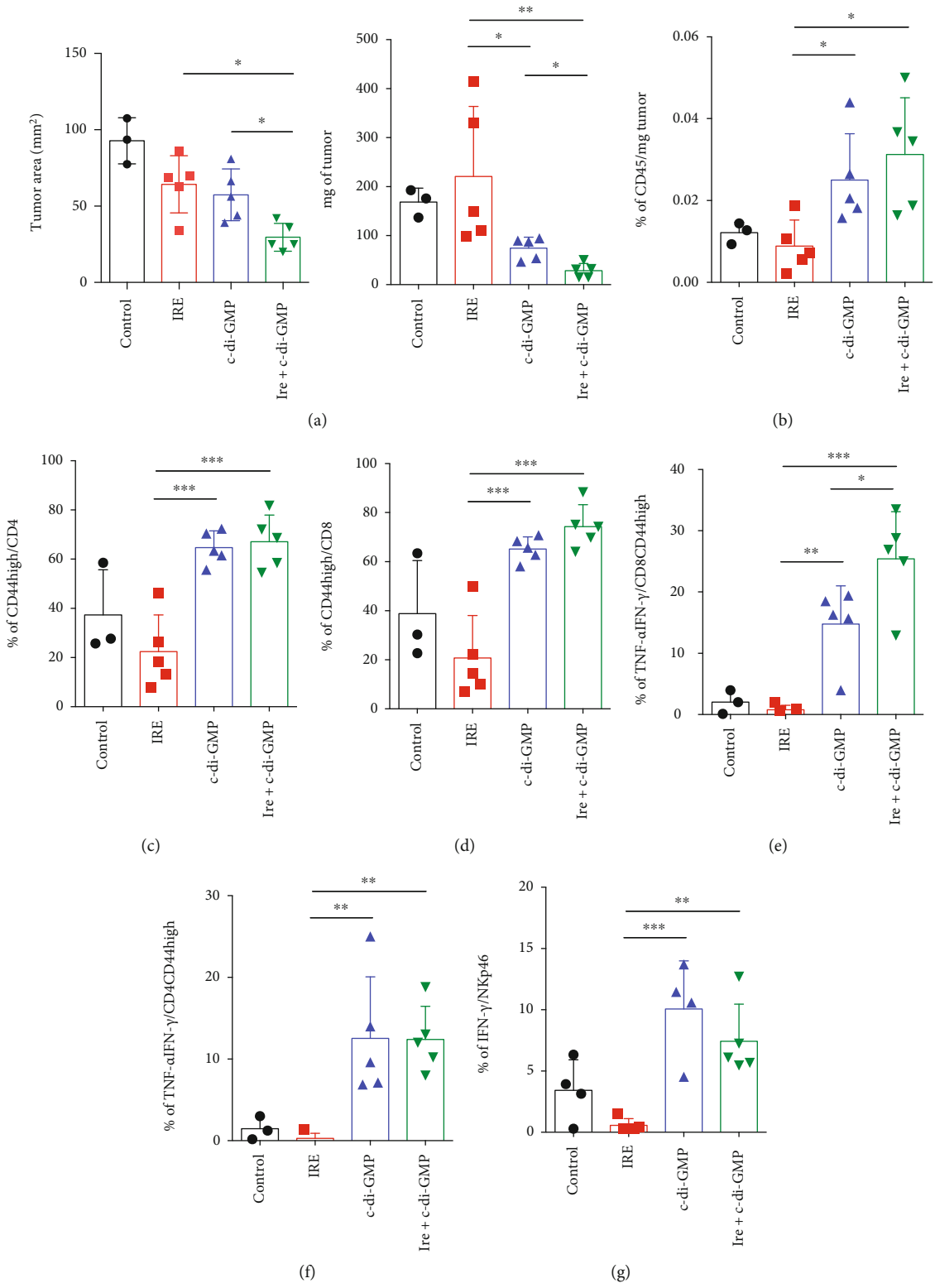

Figure 3: Phenotypic and functional analysis of intratumor T lymphocytes in mice bearing PM299L tumors. Mice were challenged with PM299L tumor cells s.c. and at days 7-10, when tumors reached 5 mm in diameter, they were treated i.t. as indicated and sacrificed seven days later for phenotypic analysis of tumor-infiltrating lymphocytes. (a) Tumor area (measured with a caliper) and tumor weight measured in each individual mice the day of sacrifice. (b–g) Phenotypic and functional analysis of tumor-infiltrating T lymphocytes and NK cells measured by flow cytometry using the indicated antibodies. One-way ANOVA with Tukey's multiple comparison test. $p < 0.05$; $**p < 0.01$; $***p < 0.001$.

cGAS–STING–TBK1 signaling pathway for their clinical use as a new immune stimulatory therapy. While multiple new generation STING agonists are being advanced into clinical development (reviewed in [19]), data from initial phase I clinical trials showed that STING agonists alone elicited modest therapeutic efficacy [34]. This poor efficacy was in part due to their poor pharmacokinetic profile. The anionic properties of STING agonists reduce their membrane permeability, limiting their entry into the cytosol and the activation of the STING pathway. Moreover, systemic delivery of STING agonists for cancer therapy can induce off-target generalized inflammation or autoimmunity, since they do not preferentially localize to tumor tissue. We hypothesized that the anionic charge of the STING agonist c-di-GMP could facilitate its internalization into the tumor cells in vivo through the nanopores in the cell membrane caused by the IRE procedure, as it has been proposed by other means, such as the use of liposomal encapsulation [35]. Moreover, dead tumor cells loaded with STING agonists could be engulfed by DC and improve their maturation and the induction of a tumor-specific T cell immune response. In this scenario, we proposed the combination of intratumoral injection of c-di-GMP immediately followed by IRE as a more efficient antitumor therapy. We found that combination of IRE and c-di-GMP was able to delay tumor growth in two murine tumor models. We observed a significant delay in tumor growth B16.OVA melanoma and PM299L HCC tumor models. Interestingly, female mice responded more efficiently to combined therapy than male mice. This result is in agreement with previous reports showing that female mice respond better to immunotherapy [36]. Gender influence on cancer immunotherapy has been recently reviewed by Irelli et al. [37].

Image-guided locoregional therapies have increased substantially the overall 5-year survival of patients with liver cancers. However, new and more efficient treatment approaches are warranted to further improve treatment outcomes. The combination of local and systemic therapies is being actively studied to increase response rates (reviewed in [38]). Combination of locoregional therapies, such as local radiation, thermal ablations, or transarterial chemoembolization, with the systemic administration of immune checkpoint inhibitors has demonstrated increased antitumor immune response and constitutes a promising combination [39–41]. Intratumor administration of oncolytic viruses in combination with anti-PD1 antibodies is currently being investigated in clinical trials (NCT03071094, NCT02509507). Combination of novel immunotherapeutic strategies with locoregional therapies is indeed a treatment concept being actively developed. Several clinical trials have been initiated to test the combination of immune checkpoint blockade and other immunotherapies plus locoregional therapies (reviewed in [42]). All these trials will shed more light on the mechanisms of action of these combined therapies and will guide clinicians in designing more effective therapeutic strategies for each patient.

Our results show that the combination of IRE with STING agonist favors the activation of an antitumor T cell immune response compared to the single intratumoral administration of c-di-GMP or IRE treatment alone. This study have several limitations. New studies are needed to improve the efficacy of this combined therapy. An optimization of the IRE protocol, number of pulses and voltages, dose of STING agonist, delivery route, or repetitions of the therapy at different time points should be tested. In addition, a deeper analysis of immunological effects of locoregional therapies and synergies with immunomodulatory agents will help in the understanding of the mechanism of action of this combination therapy. Also, other variables such the age of animals, the type of tumors, or tumor heterogeneity, which may affect to immunotherapies [43, 44], should also be evaluated. Despite these limitations, and the difficulty of extrapolating preclinical data to clinical practice with patients, the present data could constitute the basis for clinically testing this combination therapy in refractory HCC.

Data Availability

All data supporting the results has been included in the manuscript.

Conflicts of Interest

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Authors' Contributions

Aritz Lasarte-Cia and Teresa Lozano have contributed equally to this work and share first authorship.

Acknowledgments

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Supplementary Materials

Figure S1: phenotypic and functional analysis of intratumor T lymphocytes in mice bearing PM299L tumors. Mice were challenged with PM299L tumor cells s.c., and at days 7-10, when tumors reached 5 mm in diameter, they were treated i.t. as indicated and sacrificed seven days later for phenotypic analysis of tumor-infiltrating lymphocytes. Phenotypic and functional analysis of tumor-infiltrating T lymphocytes measured by flow cytometry using the indicated antibodies. One-way ANOVA with Tukey's multiple comparison test, $p < 0.05$; $**p < 0.01$; $***p < 0.001$. *(Supplementary Materials)*

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Review Article

Hepigenetics: A Review of Epigenetic Modulators and Potential Therapies in Hepatocellular Carcinoma

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Hepatocellular carcinoma is the fifth most common cancer worldwide and the second most lethal, following lung cancer. Currently applied therapeutic practices rely on surgical resection, chemotherapy and radiotherapy, or a combination thereof. These treatment options are associated with extreme adversities, and risk/benefit ratios do not always work in patients' favor. Anomalies of the epigenome lie at the epicenter of aberrant molecular mechanisms by which the disease develops and progresses. Modulation of these anomalous events poses a promising prospect for alternative treatment options, with an abundance of felicitous results reported in recent years. Herein, the most recent epigenetic modulators in hepatocellular carcinoma are recapitulated on.

1. Introduction

Hepatocellular carcinoma (HCC) is a notoriously aggressive cancer with high global prevalence rates and is the next most common perpetrator of cancer-related death following pulmonary carcinomas, with annual mortality rates of the order of 800,000 deaths [1]. HCC develops in a backdrop of a chronic liver disease that ultimately results in liver fibrosis and cirrhosis, which are consequential HCC risk factors. Hepatitis C and B, aflatoxins, alcoholic liver disease, and nonalcoholic steatohepatitis are all commonly encountered chronic inflammatory hepatopathologies that predispose to HCC. Depending on the etiology, disparate molecular dysregulation patterns arise, all converging on promoting malignancy. The loss of cell cycle restraints, incapacity to senesce, and disarrayed apoptosis [2] are among such dysregulated mechanisms, which could well be the result of genetic as well as epigenetic alterations.

The epigenome constitutes heritable features of the genetic material out with the DNA sequence. Specific epigenetic patterns are important for the maintenance of cellular integrity and gene expression patterns associated with health. In this capacity, the epigenetic fingerprint functions to guarantee proper and timely expression of genetic information, and its alteration aggravates pernicious cellular changes, many of which predispose to cancer [3]. Herein, a compendium of the most recent work addressing epigenetic modulators in the context of HCC is presented.

1.1. What Is Epigenetics? Epigenetics is a term that was first coined by Conrad Waddington, and it literally means "above genetics" [4]. It entails changes to cellular phenotypes, which are not dependent on alterations of the genetic code (DNA sequence). However, unanimity regarding the definition of epigenetics has thus far been elusive, and debates in this regard have been inconclusive at best [5].

As previously mentioned, the most recognized of epigenetic mechanisms involve chromatin remodeling. Chromatin is the macromolecule by virtue of which the genetic material can be packed inside cells' nuclei. It is composed of nucleosomes: DNA wound around histone protein octamers. In its compact form, the heterochromatin, the genetic material is relatively inaccessible for replication and the genes within are largely silent. The euchromatin on the other hand is a relaxed form of chromatin where the DNA is more accessible and genes are more or less actively expressed [5]. It can thus be easily concluded that regulation of chromatin condensation plays a role in regulating gene expression and the

resulting phenotypes. Chromatin-modifying enzymes are key players in effecting such restructuring and subsequent modifications to DNA and the histone scaffolding on which it is wound.

CpG islands are clusters of CpG dinucleotides predominantly found in the promoter regions of genes. Generally, methylation of the 5-carbon in the cytosine of these CpG islands shields the promoter from the transcription machinery to the end result of a controlled gene expression. On the other hand, demethylation of these regions within gene promoters allows for the recruitment of the transcription machinery and the gene is essentially "on." Such functionality is predominantly reserved for DNA methyltransferases. That being said, promoters containing CpG islands account for only 70% of the promoters in the genome. Interaction with the remaining 30% is orchestrated by modifications to the histone proteins, regulated—to a large extent—by histone deacetylases [5]. The disruption of these mechanisms can thus lead to aberrations in gene expression, which in many cases can initiate or promote oncogenesis. For example, the promoters of genes, which are normally turned off, are usually found hypomethylated in cancer.

1.2. Epigenetic Modulators. Options for epigenetic therapies in HCC can be enumerated as follows: inhibitors of DNA methyltransferases, regulators of histone methyltransferases, demethylases, acetyltransferases, and—most prominently—deacetylases. Another major class of epigenetic modulators is represented in noncoding RNAs. Below, the most eminent and clinically established classes are explored comprehensively to afford an encyclopedic overview of the current status of epigenetic recourse for HCC therapy. However, due to scarcity of data, several agents such tacedinaline, romidepsin, some helicases, and other enzymes viz. acireductone dioxygenase 1 are not discussed.

2. DNA Modifications

2.1. DNA Methyltransferases (DNMTs). The implication of epigenetic changes in HCC, specifically aberrant patterns of DNA methylation, has recently been recognized as a primary contributor to disease onset and progression [6]. As a consequence of such epigenetic anomalies, key tumor suppressors may be silenced or oncogenes activated, resulting in the initiation of tumorigenesis. DNA methylation is mediated by a conserved class of catalytic proteins known as *DNA methyltransferases* (DNMTs). DNMTs are key players of the epigenome. DNMTs come in two primary categories, maintenance (DNMT1) and *de novo* DNMTs (DNMT3a and DNMT3b) [7]. Although the distinction is not absolute, it does hold contemporarily. *DNMT1*, *DNMT3a*, and *DNMT3b* function by catalyzing the transfer of a methyl group from S-adenosyl-L-methionine, the universal methyl donor to a $5'$-cytosine on DNA [8]. Moreover, several other DNMTs do exist (such as *DNMT2* and *DNMTL*); however, they remain relatively undefined despite having demonstrated a role in HCC [9].

Despite the widely suggested distinction that *DNMT1* functions as the maintenance methyltransferase and *DNMT3a* and *DNMT3b* mediate *de novo* methylation (predominantly during embryonic development), the notion has been challenged as of late, with *DNMT1* recognized as a contributor to *de novo* methylation while maintenance functions are mediated by *DNMT3a* and *DNMT3b* in concert with *DNMT1* [10]. Notwithstanding the above-mentioned classification, these enzymes do not function individually and their interaction is crucial to the creation and maintenance of appropriate methylation patterns. The alteration of such coordination has in fact been associated with cancer development [11].

2.2. DNMT1. *DNMT1* is the most common subtype in adult cells [12]. Normally, *DNMT1* functions to maintain methylation patterns of CpG sites within promoters. This is achieved by *DNMT1* accessing hemi-methylated DNA during replication, priming the daughter unmethylated strand for methylation. However, anomalous DNMT-mediated methylation jeopardizes typical gene expression patterns as a result of increased or decreased accessibility of CpG-rich promoters. HCC and its adjacent tissues have demonstrated notably different DNA methylation patterns [6]. Where the noncancerous neighboring tissues display uniform and stable methylation patterns, HCC exhibits a marked heterogeneity. According to the reported results, HCC tissues manifest reduced methylation of CpG regions. Table 1 shows a snippet of the reported signature of methylated genes in HCC, which is reportedly capable of differentiating HCC samples from neighboring tissues. A former study showed that DNA methylation of CpG island-associated promoters silenced gene expression and defined 222 drivers of epigenetic changes exhibiting this negative correlation. A preponderance of these candidate drivers was found to be enriched in inflammatory responses, a number of metabolic processes, and oxidation-reduction reactions. A set of reliable and robust candidates was also defined (Table 1).

Neurofilament, *heavy polypeptide* (*NEFH*) and *sphingomyelin phosphodiesterase 3* (*SMPD3*) were also defined as tumor suppressor genes that were hypermethylated and silenced in HCC [13]. The results obtained from the gain of function experiments revealed diminished cellular proliferation, whereas those of knockdowns restored tumor invasiveness and migratory capacities. Conversely, hypomethylation of the fetal promoters of the oncogene, *IGF2*, gave way to its overexpression, imparting virulent phenotypes [14]. DNA methylation has also been inculpated in the dysregulation of several long noncoding RNAs (lncRNAs), which have been awhile associated with HCC. The histone methyltransferase *enhancer of zeste homolog 2* (*EZH2*), which catalyzed the trimethylation at lysine 27 of histone H3, has been proven to silence *TCAM1P-004* and *RP11-598D14.1*: two tumor-suppressing long noncoding RNAs [15]. This has been supposed to be assisted by *Yin Yang 1* (*YY1*), which purportedly aids in recruiting *EZH2* to promoters of target genes [16]. The downregulation of these lncRNAs correlated with tumor progression owing to the inhibition of their moderation of the *mitogen-activated protein kinase* (*MAPK*), *tumor protein p53* (*p53*), and *hypoxia-inducible factor 1-alpha* (*HIF1-α*) pathways [15]. As would be expected, upregulation of histone methyltransferases might just be the driver for neoplastic

Table 1: Aberrant methylation patterns in hepatocellular carcinoma (HCC). A comprehensive list of genes, which were dysregulated in HCC due to aberrant methylation patterns.

Gene	Methylation pattern	Ref.
ACSL4	Hypomethylation	
ALDH3A1	Hypomethylation	[217]
APOA5	Hypermethylation	
CLDN15	Hypomethylation	
CDKN2A	Hypermethylation	[6]
CYP7A1	Hypomethylation	[217]
DEFB119	Hypomethylation	[6]
DPP6	Hypomethylation	
ENDOD1	Hypermethylation	
EZR	Hypermethylation	[217]
GLUL	Hypomethylation	
GZMB	Hypomethylation	[6]
MIR21	Hypomethylation	[218]
Myo1g	Hypermethylation	[219]
NEFH	Hypermethylation	[13]
NKX3-2	Hypermethylation	
NDRG2	Hypermethylation	[6]
PDE1A	Hypomethylation	
PHYHD1	Hypermethylation	[217]
PRH2	Hypermethylation	[6]
RASSF1A	Hypermethylation	[220]
RP11-598D14.1	Hypermethylation	[15]
SCAND3	Hypermethylation	[219]
SPP1	Hypomethylation	[217]
SPRR2A	Hypomethylation	[6]
SLC25A47	Hypermethylation	[6]
SLC25A47	Hypermethylation	[217]
SLC39A12	Hypomethylation	[6]
SMPD3	Hypermethylation	[13]
SFN	Hypomethylation	[217]
SGCA	Hypomethylation	[6]
TBX4	Hypermethylation	
TCAM1P-004	Hypermethylation	[15]
TKT	Hypomethylation	[217]
VTRNA2-1	Hypermethylation	[221]
ZPBP	Hypermethylation	[6]

ACSL4: Acyl-CoA Synthetase Long Chain Family Member 4; *ALDH3A1*: Aldehyde Dehydrogenase 3 Family Member A1; *APOA5*: Apolipoprotein A5; CLDN15: Claudin-15; *CDKN2A*: cyclin-dependent kinase inhibitor 2A; *CYP7A1*: Cytochrome P450 Family 7 Subfamily A Member 1; *DEFB119*: Defensin β 119; *DPP6*: Dipeptidyl peptidase 6; *ENDOD1*: Endonuclease Domain Containing 1; *EZR*: Ezrin; *GLUL*: Glutamate-Ammonia Ligase; *GZMB*: Granzyme B; *MIR21*: microRNA-21; *Myo1g*: Myosin 1g; *NDRG2*: N-myc downstream-regulated gene family member 2; *NEFH*: Neurofilament, heavy polypeptide; *NKX3-2*: NK3 Homeobox 2; *PDE1A*: Phosphodiesterase 1A; *PHYHD1*: Phytanoyl-CoA Dioxygenase Domain Containing 1; *PRH2*: Proline-rich protein HaeIII subfamily 2; *RASSF1A:* Ras association domain family 1 isoform A; SCAND3: SCAN domain containing 3; *SFN*: Stratifin; *SGCA*: α-sarcoglycan; *SLC25A47*: Solute Carrier Family 25 Member 47; *SLC39A12*: Solute carrier family 39 member 12; *SMPD3*: sphingomyelin phosphodiesterase 3; *SPP1*: Secreted Phosphoprotein 1; *SPRR2A*: Small proline-rich protein 2A; *TBX4*: T-box 4; *TKT*: Transketolase; *VTRNA2-1*: Vault RNA 2–1; *ZPBP*: Zona pellucida binding protein.

events, given their downstream action on key promoters. By way of instance, *SET domain bifurcated histone lysine methyltransferase 1* (*SETDB1*), an H3K9-specific methyltransferase, has been reported to exhibit the most substantial increase in HCC in comparison to other epigenetic regulators [17]. *SETDB1* was shown to owe its overexpression in HCC to a gene duplication event, with an additional copy of chromosome 1q21 [17]. However, other anomalous events were discovered to contribute to its elevated levels, such as regulation by microRNAs (discussed below), or transcriptional activation such as this mediated by *specificity protein 1* (*SP1*) [17].

2.3. DNMT3. Contrary to *DNMT1*, *DNMT3a* and *DNMT3b* do not recognize hemimethylated DNA. They do not produce or maintain particular patterns of methylation [18], and they are not specifically associated with replication sites [19] as *DNMT1*. Rather, they mediate *de novo* methylation as mentioned previously. Additionally, it has been assumed that these DNMTs employ mechanisms different from *DNMT1* to access the heterochromatin [20], given the fact that they were found not to be associated with replication sites.

DNMT3 has been implicated in hepatocarcinogenesis. It has been expressly associated with hypermethylation of promoters controlling 22 tumor suppressor genes [21]. *DNMT3b* also exhibited a 4-fold increase of expression in HCC when compared to healthy livers, which correlated with poorer prognosis [21], which corroborates assumptions that *DNMT3* subtypes become overexpressed in cancer after having been downregulated postcellular differentiation [22].

In HCC of HBV etiology, the normally silenced *metastasis-associated protein 1* (*MTA1*) gene was upregulated by recruitment of *DNMT3a* and *DNMT3b* leading to hypomethylation of its promoter and increasing the tumor metastatic disposition [23]. Additionally, *DNMT3b* was elsewhere reported to be overexpressed by *telomerase reverse transcriptase* (*TERT*) in HCC. The resulting anomalous methylation patterns prompted activation of *AKT* [24]. Apart from its methylating capacity, *DNMT3b* was found to directly target *metastasis suppressor 1* (*MTSS1*), by direct binding to its promoter [25].

The implication of *DNMT3a* in HCC has also been corroborated. In a study by Zao et al., *DNMT3a* knockdowns displayed arrested cellular proliferation. Microarray analysis revealed concomitant upregulation of 153 genes, the preponderance of which bears CpG islands in their promoters. Among these activated genes was the tumor suppressor *PTEN* gene [26]. Moreover, *DNMTa* guided a conjectured distinction in the epigenetic dysregulation between different forms of liver cancer, where nonfibrolamellar HCC displayed significantly higher levels of *DNMTa* compared to the fibrolamellar variant [27]. This discrepancy was suggested to betray divergent epigenetic mechanisms in different HCC subtypes.

2.4. DNMT3L. Structurally similar and functionally complementary to *DNMT3a* and *DNMT3b* is *DNMT3L*, which, despite lacking intrinsic catalytic activity, enhances the binding of the former to S-adenosyl-L-methionine, the donor of

Table 2: DNA methyltransferase (DNMT) inhibitors in HCC. The table shows the most prominent DNMT inhibitors, the changes in the targets of the inhibited DNMTs, and the resulting effects on the tumor.

DNMT inhibitor	DNMT targets affected	Effect	Ref.
5-Azacytidine	*SLC10A1*, *CYP3A4*, *ALB*, and *miR-122*	Inhibits tumor growth	[29]
Decaitabine	*p16INK4A* (activation)	G1 cell cycle arrest	[35]
	PRSS3 (activation)	Inhibits proliferation and migration	[36]
Guadecitabine (SGI-110)	*DLEC1*, *RUNX3*, and *p16INK4A*	Inhibits tumor growth	[38]
Zebularine	*CDK2*, *Bcl-2*, and phosphorylation of *Rb* (inhibition) and *p21WAF/CIP1* and *p53* (activation)	Inhibits proliferation and induces apoptosis	[42]
SGI-1027	*Bcl-2* (inhibition) and *BAX* (activation)	Induces apoptosis	[222]
CM-272	*E-cadherin*, *CYP7A1*, *FBP1*, *GNMT*, and *MAT1A* (activation)	Inhibits proliferation and decreases adaptation to hypoxia	[223]
EGCG (Y6)	*P-gp* and *HIF1-α* (inhibition)	Inhibits proliferation and reverses doxorubicin-resistance	[53]
Genistein	*CYP1A1*, *CYP1B1*, and *p-AMPK* (activation) and *CYP26A1* and *CYP26B1* (inhibition)	Inhibits proliferation (at a 10-40 μM concentration) and induces apoptosis	[44]

ALB: albumin; *BAX*: Bcl-2-like protein 4; *Bcl-2*: B-cell lymphoma 2; *CDK2*: cyclin-dependent kinase 2; *CYP1A1*: cytochrome P450 1A1; *CYP1B1*: cytochrome P450 1B1; *CYP26A1*: cytochrome P450 26A1; *CYP26B1*: cytochrome P450 26B1; *CYP3A4*: cytochrome P450 3A4; *CYP7A1*: cholesterol 7*α*-hydroxylase-1; *DLEC1*: deleted in lung and esophageal cancer 1; *FBP1*: fructose-1,6-bisphosphatase; *GNMT*: glycine-N-methyl transferase; *HIF1-α*: hypoxia-inducible factor 1-*α*; *MAT1A*: methionine-adenosyltransferase 1A; *p16INK4A*: cyclin-dependent kinase inhibitor 2A; *p21WAF/CIP1*: cyclin-dependent kinase inhibitor 1; *p53*: tumor protein p53; *p-AMPK*: phosphorylated AMP-activated protein kinase; *P-gp*: P-glycoprotein 1; *Rb*: retinoblastoma; *RUNX3*: RUNX Family Transcription Factor 3; *SLC10A1*: sodium/bile acid cotransporter.

the methyl group. Understanding the role of *DNMT3L* in full requires further analysis [28].

Given all of the above, it is clear that modifying any of these anomalies could potentially serve as a therapeutic modality in HCC. Below the major DNMT inhibitors with reported activity in HCC are outlined.

2.5. DNMT Inhibitors. Herein, the most prominent inhibitors of DNMT in HCC are outlined. Despite the fact that—in many instances—DNMT inhibitors may not be selective for one subtype over the other, the following is reported according to what the original account relayed. DNMT inhibitors are summarized in Table 2.

2.6. 5-Azacytidine. 5-Azacytidine (5-AZA) is a synthetic analog of the nucleoside cytidine and an established inhibitor of *DNMT1*, marketed under the name Vidaza. In the context of HCC, treatment with 5-AZA conduced to tumor regression and a shift to a more differentiated phenotype, which was associated with regional demethylation of CpG regions upstream of the liver-specific genes *SLC10A1*, *CYP3A4*, *ALB*, and *miR-122*, which were downregulated pretreatments [29]. Additionally, this epigenetic modulation boosted the effects of sorafenib. 5-AZA triggered demethylation of 5-hydroxymethylcytosine (5hmC) via the *ten-eleven translocation proteins 2 and 3* [30]. *DNMT1* inhibition by 5-AZA was also found to synergize with immunotherapy via encouraging trafficking of T-cells to the tumor microenvironment secondary to a 5-AZA-induced upregulation of chemokine genes [31]. 5-AZA has been determined to be potentiated by sundry supplementation, such as vitamin C [32] and alendronate [33]. More recently, 5-aza-2′-deoxycytidine (5-Aza-CdR), a derivative of 5-AZA, was reported to downregulate *DNMT1*, *DNMT3a*, and *DNMT3b* [34].

2.7. Decitabine. Decitabine (5-aza-2′-deoxycytidine) is another analog of cytidine that also acts by blocking *DNMT1*. Decitabine was reported to demethylate the promoter of the *p16INK4A* gene, the product of which functions to regulate the cyclin-dependent kinases 4 and 6, leading to an upsurge of *p16INK4A* transcripts with ensuing G1 cell cycle arrest and a rise of the senescence-associated β-galactosidase [35]. Expression levels of *PRSS3* were also reported to rise in decitabine-treated cells [36]. The desilencing of *PRSS3* decelerated cellular proliferation due to inhibition of two cyclin/CDK complexes and downshifted migration through silencing *matrix metalloproteinase 2* (*MMP2*). A phase I/II clinical trial [37] scrutinized the efficacy of decitabine and its safety in advanced HCC. Western blots from patients' peripheral blood mononuclear cells (PBMCs) indicated decreased levels of DNMT1 in decitabine-treated participants.

2.8. Guadecitabine. Guadecitabine is a dinucleotide derivative of decitabine in which the latter is attached to a deoxyguanosine is by a phosphodiester bridge. Guadecitabine is commonly designated as SGI-110 and exhibits a more sustained systemic effect than its parent compound. Demethylation and activation of the tumor suppressor genes *DLEC1*, *RUNX3*, and *CDKN2A* were observed following SGI-110 treatment of Huh7 and HepG2 cells. Although its demethylating effects were compromised in the presence of the histone H2A variant, macroH2A1, SGI-110 was still capable of restricting tumor growth, unlike decitabine [38]. Potentiation of the cytotoxicity of the platinum-based antineoplastic oxaliplatin was reported when a pretreatment of SGI-110 was coadministered [39]. The mechanistic basis of such a sensitization involves counteracting the extensive methylation of targets within the *Wnt/EGF/IGF* signaling loop.

2.9. Zebularine. In HepG2 cells cultured at high densities, zebularine, a more stable and less toxic analog of 5-AZA [40], demonstrated a progressive escalation of expression of differentiation-associated genes and fomented apoptosis. shRNA-induced *DNMT1* knockdown annulled these effects [41]. Paradoxically, contrary reports indicated that zebularine had negligible influence on DNA methylation in the same cell line [42]. Despite the previous report, zebularine did affect several cytotoxic events, which have been attributed to mechanisms other than DNMT inhibition. Zebularine was found to inhibit *histone deacetylases* (*HDACs*) alongside DNMT genes in LS 174T cells [43]. *DNMT1*, *DNMT3a*, and *DNMT3a* as well as Class I HDACs and Class II HDACs were downregulated with a concomitant elevation in the expression of *p21Cip1*/*Waf1*/*Sdi1*, *p27Kip1*, and *p57Kip2* on treatment with zebularine, albeit to a more modest extent in comparison with trichostatin A. In the same study, it was observed that both agents acted synergistically to substantially increase apoptosis. It would thus seem propitious to examine these regulatory loops more closely in HCC.

2.10. Genistein. Genistein (GE) is an isoflavone derived from soybean and is characterized by its propensity to bind the estrogen receptor. GE upregulated cytochromes *1A1* and *1B1* in HT29 cells and downregulated cytochromes *26A1* and *26B1* [44]. In Hep3B cells, GE increased levels of phospho-*AMPK*, which mitigated inflammatory processes and consequent liver damage [45]. In concert with trichostatin A (TSA), GE restored the expression of the DNA methyltransferases *DNMT1*, *DNMT3a*, and *DNMT3b* in HepG2 cells [46]. GE exhibited biphasic effects at different concentration ranges, where at a low concentration of 1 μM, it encouraged cellular growth, while at higher concentration within the range of 10-40 μM, GE had antiproliferative effects. Proapoptotic effects were evident at all concentrations, unlike TSA, whose effects were observable only following a 3-day long treatment [47].

2.11. Epigallocatechin-3-Gallate (EGCG). EGCG is the most abundant catechin in green tea that—among other flavonoids and catechins—has repeatedly been reported to possess tumor chemopreventive and antineoplastic effects in HCC [48]. EGCG has been shown to interact with the following amino acid residues within the catalytic domain of DNMT: P-1223, C-1225, S-1229, E-1265, and R-1309 [49, 50]. Moreover, catechol-containing polyphenols, of which EGCG is a member, inhibit DNMTs by mediating a rise in SAM O-methylation via catechol-O-methyltransferase. Alternatively, SAM levels were increased following disruption of the folate cycle secondary to dihydrofolate reductase inhibition by catechol-containing polyphenols. Direct inhibition of DNMTs by this class of compounds can also occur regardless of the methylation pattern [49, 50].

Additionally, EGCG has been shown to mediate a metabolic shift away from glycolysis in HCC cells, thereby promoting apoptosis and stunting cellular proliferation [51]. Mechanistically, this action has been attributed to its suppression of phosphofructokinase activity, whereby cellular stress is effected, ultimately culminating in programmed cell death. What is more, EGCG synergistically acted to ameliorate the antiproliferative effects of sorafenib [51]. Synergy between EGCG and metformin, the famous antidiabetic biguanide, has also been reported [52]. An EGCG/metformin combination therapy was associated with a significant reduction in *glypican-3*, *survivin*, *cyclin D1*, *VEGF*, and the long noncoding RNA *AF085935* and an elevation of the levels of *caspase 3* [52]. Another study examined the therapeutic effects of Y6, a chemically modified form of EGCG [53]. Again, and similar to its parent compound, Y6 efficiently curbed cellular proliferation. Additionally, it engendered a reversal of doxorubicin resistance in resistant BEL-7404 cells. The antiproliferative and antiapoptotic effects of Y6 correlated with reduced *P-glycoprotein 1* (*P-gp*) and *HIF1-α* on the mRNA and protein levels and was exacerbated in groups receiving Y6/doxorubicin combination therapy, compared to those on doxorubicin monotherapy. A compendium of studies reporting disease-modifying capabilities of EGCG in HCC can be found in a recent review by Bimonte et al. [48].

Other inhibitors of DNMT such as hydralazine, procainamide, and RG108 have been tested for their efficacy in cancer [11] but are yet to be examined as potential therapies in HCC.

3. Histone Modifications

Chromatin is formed by the assembly of nucleosomal units, which are formed by the wounding of DNA around histone proteins. For accessing of genetic information, the highly packed chromatin has to be unwound. Chromatin modifications viz. methylation and acetylation are key controllers of this stipulation and thus play a crucial role in gene expression (Figure 1).

Histone modifications comprise sundry alterations to histone proteins including methylation (histone methyltransferases and histone demethylases), acetylation (histone acetyltransferases and histone deacetylases), ubiquitination, sumoylation, and phosphorylation [54]. The disruption of any of these modification patterns entails repercussions that may very well conduce to malignancy. However, for the purpose of this review, we elected to center this discourse on histone deacetylases (HDACs) given the abundance of data and the corroborated efficiency of HDAC inhibitors in preclinical and clinical settings [55]. Other reviews can be consulted for in-depth discussion of histone modifications and their implications in cancer [56–59].

Histone acetylation is controlled by two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the acetylation of lysine residues, whereas HDACs function to remove these acetyl groups [60].

As a result of acetylation, interaction between the histone octamers and DNA is compromised due to the neutralization of the positively charged lysine residues. The weakening of this interaction gives way to a transcriptionally permissive state of chromatin. HDACs promote an opposite effect, where the euchromatin state is favored as a consequence of retrieval of the positive charges on lysine residues, restoring the histone-DNA interaction [61]. A balance between HAT

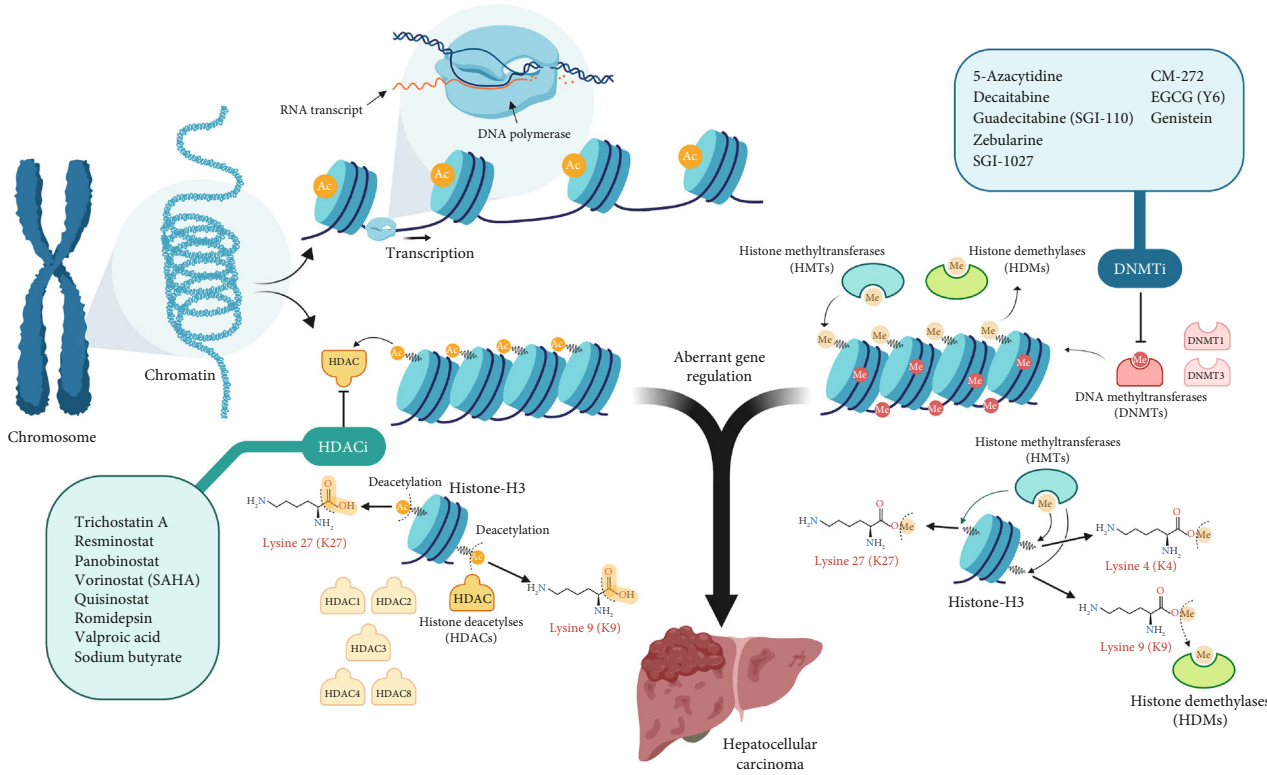

Figure 1: Epigenetic modulation of chromatin by histone deacetylation and methylation/demethylation as well as DNA methylation. The figure highlights the role of histone deacetylases (HDACs), histone methyltransferase (HMTs), histone demethylases (HDMs), and DNA methyltransferases (DNMTs) in creating the epigenetic signature observed in HCC in addition to their significance as targets for therapy. As is shown, the most common site for such modifications occurs on specific lysine residues on histone H3. "Created with BioRender."

and HDAC activity ensures the maintenance of normal patterns of gene expression, and its disruption is often noted in many malignancies including HCC [62].

3.1. HDACs. There are around 18 HDACs, many of which have been shown to deacetylate nonhistone proteins [63]. Given the above, the centrality of HDACs to chromatin accessibility and control of gene expression [64] is obvious, and assumptions that HDACs constitute tumor suppressors or target for therapy are not only well-grounded but also experimentally evident.

In HCC, dysregulation of HDACs has been multiplied reported. By way of instance, *HDAC1* and *HDAC2* were found to be overexpressed in HCC patients of Southeast Asian origin and was associated with higher rates of mortality. Inhibition of these HDACs *in vitro* inhibited cellular proliferation [65]. The upregulation of *HDAC1* and *HDAC2* was found to suppress *fructose-1,6-bisphosphatase* (*FBP1*), a key enzyme in glycolysis [66], and *HDAC2* was further reported to modulate genes involved in the cell cycle and apoptosis [67]. *HDAC3* was recently demonstrated to be centrally implicated in hepatocarcinogenesis. Following a ubiquitination event, it dissociates from the *c-Myc* promoter, whereby K9 of histone H3 (H3K9) becomes acetylated and *c-Myc* is made transcriptionally available [68]. Elimination of *HDAC3* inhibited the trimethylation of H3K9 that occurs subsequent to the *HDAC3*-mediated deacetylation of this residue, arresting the contingent double-strand break repair mechanism and resulting in the accretion of bad DNA [69].

Interestingly, HDACs were also shown to counter cell migration. Acetylation of H3K4 and H3K56 within the *Snail2* promoter was markedly reduced in EMT thanks to *HDAC1* and *HDAC3* [70]. It is worthy to note that G9a, a histone H3 lysine 9 (H3K9) methyltransferase, has been recently recognized as vital for such *Snail2*-mediated inhibition of *E-cadherin* and consequent repression of mesenchymal properties [71]. It has even been targeted for therapy by administering its inhibitor, UNC0646, in nanodiamonds, which reduced H3K9 methylation and tumor invasiveness [72].

That being said, therapeutic inhibition of HDACs may sometimes prove problematic because of interference with various pathways [56] and, as evident above, for the bidirectional functionality it has sometimes demonstrated. It is thus of essence to dedicate some efforts to better understand and characterize the complex regulatory role of HDACs so as to determine their amenability to therapeutic targeting and define in what direction should therapeutic strategies be pursued.

3.2. HDAC Inhibitors. HDAC inhibitors (HDACi) are a group of agents that are useful in resolving aberrant patterns of deacetylation, modulating chromatin accessibility, the lack of which is often an inciting factor for tumorigenesis [73]. Below the most prominent HDACis are outlined (Table 3).

Table 3: Histone deacetylase (HDAC) inhibitors in HCC. The table shows the most prominent HDAC inhibitors that have been studied in HCC, their cellular targets, and their antitumor effects.

HDACi	Target(s)	Effect	Ref.
	Hydroxamates		
Trichostatin A	*Apaf1* and *H2Aub* (activation)	Promotes apoptosis	[74]
	ULBP1/2/3 and *MICA/B* (Activation)	Inhibits tumor cell growth	[77]
Resminostat	*Caspase 9* and *cytochrome c* (activation)	Promotes mitochondrial depolarization and apoptosis	[80]
Panobinostat	*Beclin1*, *Map1LC3B*, and *p53* (activation) and *p73* nuclear translocation	Promotes autophagy	[86]
Vorinostat (SAHA)	*HIF-α* (inhibition)	Initiating tumor hypoxia	[73]
	DR5 (activation) and *c-Flip* (inhibition)	Sensitization to TRAIL-induced apoptosis	[224]
Quisinostat (±sorafenib)	*c-Caspase 3*, *c-Caspase 9*, *c-PARP*, and *Bax* (activation) and *Bcl-xL*, *Bcl-2*, *survivin*, *PI3K-p110*, *PI3K-p85*, and *p-AKT* (inhibition)	Inducing G0/G1 phase arrest and apoptosis	[225]
	Cyclic peptides		
Romidepsin	*p-Erk* and *p-JNK* (activation)	Induces cell cycle arrest in the G2/M phase and apoptosis	[226]
	Aliphatic fatty acids		
Valproic acid	*Nrf2* (inhibition)	Sensitization to proton irradiation	[94]
Valproic acid (+DOX)	*AKT*/*mTOR* (inhibition)	Increases ROS and induces autophagy	[95]
Sodium butyrate	*p-AKT* and *mTOR* (inhibition) and *CYLD* (activation)	Increases ROS and induces autophagy	[99], [76]

Bax: Bcl-2-associated X protein; *Bcl-2*: B-cell lymphoma 2; *Bcl-xL*: B-cell lymphoma extra large; *c-Caspase 3*: cleaved caspase 3; *c-Caspase 9*: cleaved caspase 9; *c-PARP*: cleaved Poly (ADP-ribose) polymerase; *CYLD*: CYLD lysine 63 deubiquitinase; *DOX*: doxorubicin; *DR5*: death receptor 5; *mTOR*: mammalian target of rapamycin; *Nrf2*: nuclear factor erythroid 2-related factor 2; *p-AKT*: phosphorylated protein kinase B; *p-Erk*: phosphorylated extracellular-signal-regulated kinase; *PI3K-p110*: phosphatidylinositol 3-kinase subunit p110; *PI3K-p85*: phosphatidylinositol 3-kinase subunit p85; *p-JNK*: phosphorylated c-Jun N-terminal kinase; *ROS*: reactive oxygen species.

4. Hydroxamates

4.1. Trichostatin A. TSA is one of the most studied hydroxamate HDAC inhibitors. Following inhibition of HDACs 1, 2, and 3 by TSA, *apoptotic protease-activating factor 1* (*Apaf1*) was determined to become upregulated, which leads to the stimulation of mitochondrial caspase-driven apoptosis of the HLE and HLF HCC cell lines [74]. TSA was also found to restore the expression level of H2Aub, an H2A posttranslationally ubiquitinated at lysine 119, which is diminished in HCC. Simultaneously, TSA modulated the rates of H3S10 phosphorylation, which were inversely correlated with H2Aub in HCC [75]. In addition to *ubiquitin-specific peptidase 21* (*ups21*), which is responsible for the downregulation of H2Aub above, *CYLD* is another (lysine 63) deubiquitinase involved in the development of HCC. Contrary to *Ups21*, it is the inadequacy of *CYLD* that is associated with malignancy. TSA was shown to raise *CYLD* mRNA and protein levels in Huh7 and HepG2 cells [76]. Overexpression of ligands of *NKGD2* was noted following TSA treatment. It thus exerted its cytotoxic effect through stimulating natural killer (NK) cells to eliminate HCC cells [77]. Alternatively, the proapoptotic activity of TSA could be modulated by regulatory RNA species such as the long noncoding RNA, *lncRNA-uc002mbe.2*, which was increased post-TSA-treatment [78]. The proposed mechanism delineates an interaction between *lncRNA-uc002mbe.2* and *heterogeneous nuclear ribonucleoprotein A2B1* (*hnRNPA2B1*) which instigates the stimulation of *p21* and reduction of phosphorylated *AKT*. TSA has been used in conjunction with other agents such as sorafenib for enhancing therapeutic outcomes [79].

4.2. Resminostat. Resminostat is a pan-HDACi (inhibits both nuclear and cytoplasmic HDACs). In HepG2, SMMC-7721 and HepB3 cells, resminostat incited mitochondrial depolarization and apoptosis via the mitochondrial permeability transition pore pathway. It also evoked the production of *caspase 9* and *cytochrome c* [80]. The cytotoxic effects of resminostat were reinforced by inhibitors of the *mammalian target of rapamycin* (*mTOR*), which has been characterized as a resistance factor of resminostat [81]. The synergistic effects of resminostat with sorafenib have been repeatedly studied. The combination proved safe and effective. Resminostat shifted the cells from a mesenchymal to an epithelial phenotype, which better sensitized the cells to subsequent sorafenib treatment [82]. That being said, further investigation into the advantage of this combination is required. While an exploratory clinical study corroborates the above observations [83], another phase I/II study refuted an added utility of resminostat supplementation over sorafenib monotherapy [84].

4.3. Panobinostat (PANB). Another potent pan-HDACi is PANB. Studies have shown that PANB affected a negative interference with DNMTs (as outlined in Table 2) and an ensuing impedance of methylation of classically hypermethylated genes, such as *APC* and *RASSF1A* [85]. PANB encouraged an increase of autophagic factors *Beclin1* and

Map1LC3B, which concomitantly presented with the appearance of quasiautophagosome clusters along with the nuclear translocation of *p53* and *p73* in HepG2 and Hep3B cells, respectively, and regulation of *DRAM1* [86]. Ingeniously, ^{18}F probes have been used as PET tracers to monitor angiogenic progression following PANB therapy, through imaging of integrin *αvβ3*. These PET scans revealed a substantially reduced uptake in HepG2 but not in HT29 neoplasm, in response to therapy in nude mice [87].

4.4. Vorinostat (VORN; SAHA). Beyond chromatin unwounding, evidences have been provided that substantiate a role of VORN in initiating tumor hypoxia. Ostensibly, VORN-mediated acetylation of *heat shock protein 90* (*Hsp90*), a chaperone of *HIF-α*, hinders its nuclear translocation and forestalls its transcriptional activity [73]. As a result, levels of several downstream hypoxia-triggered molecules come to be deficient. VORN was used as an adjuvant to a number of anticancer drugs such as oxaliplatin [88] and the *mTOR* inhibitor, sirolimus [89]. Compared to 5-aza-2′-deoxycytidine (5-Aza-CdR), VORN exhibited superior apoptotic effects which was coincident with its inhibition of *HDAC1*. However, a combination of the two achieved maximal apoptosis of LCL-PI 11 cells [34].

4.5. Belinostat. Belinostat has been studied extensively but sporadically in different cancer types, mostly on hematologic malignancies. Despite its consistently promising results, belinostat remains underinvestigated in HCC. Hereunder, most of the reports on belinostat use in HCC are summarized. A multicenter phase I/II study aimed at determining the drug pharmacokinetic and toxicity profiles constitutes one major such report. The outcomes of the study were favorable in terms of disease stabilization (assessed via histoscores) and high tolerance to the drug, which is reflected in its outspread pharmaceutical window [78]. When combined with the checkpoint inhibitors anti-*PD-1* and anti-*CTLA-4* antibodies, belinostat potentiated the latter but not the former. The synergy was credited to a drop of regulatory T cells and a boosted *IFN-γ* production by T cells in the tumor microenvironment [90]. Withal, *PD-L1* inhibition was proposed, given its observed overexpression on antigen-presenting cancer cells and its retarded expression on effector T cells. Boron-incorporating prodrugs of belinostat have been propounded for improving its potency against solid tumors [91]. The prodrug form manifested superior bioavailability. However, the efficacy of this form remains to be examined in HCC.

5. Aliphatic Fatty Acids

5.1. Valproic Acid (VPA). VPA, a class I and IIa HDACi, has a certain favorability to it, given its reasonable cost and wide safety margin. VPA demonstrated antineoplastic effects in PLC/PRF5 and HepG2 cells [92]. Moreover, VPA was shown to mediate a dissemination of its anticancer activity through its indirect modulation of cell-free DNA. This rather unique study was conducted under the hypothesis that cfDNA can mediate intercellular signaling. The cfDNA derived from VPA-treated cells induced glycolysis in naïve HepG2 cells. Subsequent analysis of the cfDNA from these cells revealed altered characteristics. As such, it was suggested that VPA treatment can be temporarily propagated across cells via their released cfDNA [93]. VPA rendered Hep3B cells more vulnerable to proton irradiation, protracting the actuated DNA damage, and promoted irradiation-mediated apoptosis [94]. Curiously, VPA increased irradiation-induced reactive oxygen species (ROS) production and silenced *nuclear factor erythroid 2-related factor 2* (*Nrf2*), which is quickly becoming a marker of radioresistance. VPA has been used in combination with doxorubicin [95] and sorafenib [96] and boosted the cytotoxic effects of cytokine-induced killer cells [97]. Recently, VPA was assessed alongside zebularine as to the effect on *Suppressor of cytokine signaling 1* (*SOCS-1*) and *Suppressor of cytokine signaling 3* (*SOCS-3*) expression [98]. Despite both suppressing cellular growth, only VPA demonstrated an apoptotic effect and correlated with an upregulation of *SOCS-1* and *SOCS-3*.

5.2. Sodium Butyrate. Butyrate is among the short chain fatty acids that are produced as a result of the anaerobic fermentation undergone by gut microbiota, and its benefits in restraining tumor growth have been documented. The sodium salt of butyrate has been explored as an epigenetic modulator in various malignancies. However, there remains a need for exploring its utility in HCC. Elevation of ROS and consequent autophagy were noted in Huh7 cells following butyrate treatment. Levels of phosphorylated *AKT* and *mTOR* were positively inhibited, which gave to a dependent rise in *ATG5*, *Beclin1*, and *LC3-II*, with subsequent assembly of the autophagosome machinery [99]. Otherwise, as noted with TSA (above), butyrate spurred on the expression of the deubiquitinase *CYLD* in Huh7 and HepG2 cells (Kotantaki & Mosialos, 2016).

6. Noncoding RNAs

6.1. MicroRNAs. MicroRNAs (miRNAs) are probably the most frequently studied biomolecules in cancer, and for a good reason. Given their integral role in gene expression manipulation, abnormal miRNome lies at the heart of the genetic dysregulation that predisposes to oncogenesis. miRNAs are encoded mostly in intergenic regions of the genome and are transcribed by RNA polymerase II. Following transcription, a primary RNA transcript forms a hairpin loop with terminal single-stranded extensions (Figure 2). Both the 5′ and 3′ extensions are cleaved off by a microprocessing complex made up of *DROSHA*, a class 2 RNase III and its accessory protein *DGCR8*, yielding what is referred to as a precursor miRNA (pre-miRNA) (Figure 2). The pre-miRNA is exported to the cytoplasm shuttled through nuclear pores by the transporter *exportin 5* (Figure 2). In the cytoplasm, the pre-miRNA is recognized by the *TRPB2*-bound enzyme *Dicer*, another RNase III, which clips off the loop, producing a double-stranded miRNA (ds-miRNA or miR/miR∗ duplex) (Figure 2). The Argonaut protein, *Ago2*, interacts with *Dicer* to bind the ds-miRNA, unwinding the miRNA duplex, releasing the passenger strand that is degraded and retains the guide strand (Figure 2), which is

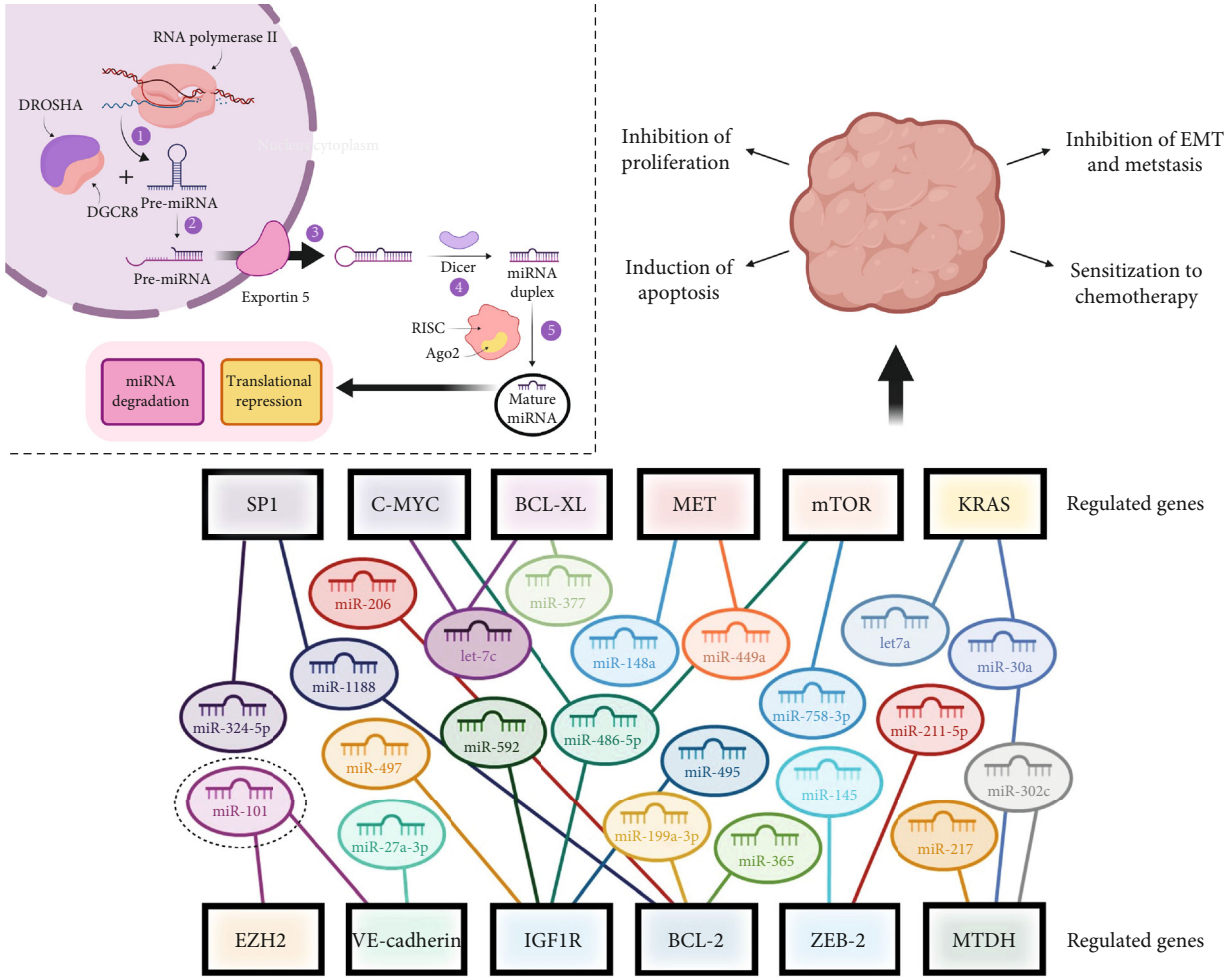

Figure 2: A schematic showing a network of several miRNAs with converging regulatory pathways in HCC therapy. The figure shows miRNAs sharing a common target as well as targets regulated by more than one miRNA. The therapeutic effects associated with all of the microRNAs in the illustrated panel correlate with their upregulation, except for miR-101 (marked). "Created with BioRender."

15-25 nucleotides long [100, 101]. Along with *Ago2*, the guide strand interacts with a group of proteins forming the *RNA-induced silencing complex* (*RISC*) which constitutes the active silencing species. Complementarity with the 3′ UTR of target mRNAs determines which are marked for silencing, which is further reinforced by near-perfect complementarity of the mRNA with the miRNA seed sequence. The bound mRNA may be degraded or its translation impeded, turning off the mRNA-encoding gene. Hereinafter, some of the most therapeutically bioactive miRNAs are explored.

6.2. miR-126. miR-126 was shown to target *EGFL7* and *VEGF* in HCC tissues, lowering their expression [102]. Gain of function studies demonstrated that this regulatory mechanism resulted in significant reduction of tumor size and weight as well as a decreased microvascular density of transplanted neoplasms. Other studies further corroborated the antiangiogenic role of *miR-126*. *miR-126*-transfected HepG2 cells were transplanted in nude mice in parallel with a control group receiving a transplant of nontransfected cells. Postresection analysis revealed lower VEGF expression levels in the *miR-126* group compared with controls as well as relatively reduced tumor volumes [103]. Du and colleagues [104] reported similar findings for the 3p arm of *miR-126*. According to the results of their experiments, *miR-126-3p* gain of function inhibited expansion of tumor vasculature and reduced microvascular density and capillary tube formation. *Low-density lipoprotein receptor-related protein 6* (*LRP6*) and *phosphoinositide-3-kinase regulatory subunit 2* (*PIK3R2*) were identified as the direct targets, and their silencing occasioned similar effects to those brought about by overexpression of *miR-126-3p*. Beyond its effects on tumor vascularization, *miR-126* has manifested antiproliferative and antiapoptotic functionalities. Zhao et al. [105] reported *sex-determining region Y-box 2* (*SOX2*) as a putative target of *miR-126*. *miR-126* mimics correlated with downregulated levels of *SOX2* and subsequent cell cycle arrest and apoptosis in HepG2 cells. In addition to the above, *miR-126* repressed metastatic capability of HCC. A negative correlation between *miR-126* and *ADAM metallopeptidase domain 9* (*ADAM9*) has been established in hepatitis B virus-related HCC [106]. Upregulation of *miR-126* attenuated *ADAM9* expression and consequently inhibited tumor migration and reduced instances of metastases. Ectopic expression of *miR-126* was

associated with failure of *miR-126*-trasnfected SMMC-7721 cells to achieve pulmonary colonization *in vivo* [107]. The *miR-126-3p*/*PIK3R2*/*LRP6* regulatory loop mentioned above has also been proven to result in the suppression of cellular migration, ECM invasion, and tumor metastasis [104].

6.3. miR-148a. *miR-148a* has recently been shown to post-transcriptionally regulate the expression of *transferrin receptor 1* (*TFR1*) [108]. Given the negative correlation observed, an increase in *miR-148a* levels is surmised to downregulate *TLR1* in HCC, resulting in reduced uptake of transferrin-bound iron by the cancer cells, which consequently leads to a drop in cellular iron levels, suppressing proliferation. The closely related *miR-148b* is purported to directly target *Rho-associated protein kinase 1* (*ROCK1*) to similar antiproliferative effects [109]. Other endeavors indicated that *miR-148a* mimics might be implicated in the regulation of hepatocytic differentiation via regulating the *IKKα*/*NUMB*/*NOTCH* pathway [110]. Furthermore, *miR-148a* positively correlated with the expression of *E-cadherin* and downregulated mesenchymal markers, i.e., *vimentin*, *fibronectin*, and *N-cadherin* in hepatoma cells, by binding and inhibiting *Met* and attenuating its downstream signaling, ultimately resulting in decreased nuclear accumulation of *SNAIL* [111]. As such, *miR-148a* was effective in discouraging EMT and suppressing pulmonary metastasis. A number of studies sought to examine the role of microRNAs in regulating hepatic stellate cells (HSCs), to outstanding outcomes. *miR-148a* was shown to target and inhibit *growth arrest-specific gene 1* (*Gas1*) mRNAs, thwarting Hedgehog signaling and preventing biogenesis of autophagosomes, which manifested as enhanced autophagy and apoptosis of HSCs [112]. Interestingly, *miR-148a* itself has been shown to be epigenetically regulated in HCC. By virtue of its hypermethylated CpG island, *miR-148a* is typically silenced in HCC cell lines [113]. Ironically, *DNMT1*, an established target of *miR-148a*, is the DNA methyltransferase that mediates such hypermethylation. *DNMT1* is upregulated in HCC, and thus, it downplays its primary regulator by a negative feedback loop. Fortunately, ectopic expression of *miR-148a* abrogates the inhibitory effects of *DNMT1*, permitting its regulatory role to take effect.

6.4. miR-199a. *miR-199a-3p* prompted a diminution of malignant nodular size and numbers in a transgenic mouse model that is prone to developing HCC, coinciding with a downregulation of its putative targets: *p21 activated kinase 4* (*PAK4*) and *mTOR*, and hence a drop in the levels of *FOXM1*, replicating effects observed following treatment with sorafenib [114]. Targeted delivery of *miR-199a-3p* to neoplasms in nude mice displayed similar auspicious outcomes. Mimics of the 3p arm of *miR-199a* were encapsulated in bionic acid- (BA-) functionalized peptide-based nanoparticles (NPs). Hepatospecific delivery was achieved through the high affinity interaction between BA and the asialoglycoprotein receptors, which are overly expressed in HCC cells. Mirroring *mTOR* inhibition *in vitro*, apoptotic and antiproliferative events were noted, following IV administration of the NPs [115]. Preceding *in vitro* analysis had additionally exposed an upregulation of *PUMA* secondary to a rise in *ZHX1* levels, concurring with repressed growth. Increased cell death was paralleled by *Bcl2* tapering off and accretion of *cleaved caspase 3* and *Bax* [116]. Both arms of *miR-199a* positively modulated *E-cadherin* through inhibition of its *Notch1*-mediated suppression [117], which also suggests a role for *miR-199a* in checking EMT. *miR-199a-5p* was also shown to restrain metastatic disposition by silencing *Snail1* [118]. The biotherapeutic activity of the 5p arm extends well beyond its regulation of *E-cadherin*. Upwards of EMT, introducing *miR-199a-5p* stifled *clathrin heavy chain* (*CTLC*) expression arresting cellular growth *in vitro* and xenograft mice models [119]. Moreover, *VEGF*-initiated cell proliferation was reportedly halted posttreatment with *miR-199a-5p*, thanks to its modulation of the *nitroreductase*, *NOR1* [120].

6.5. miR-503. Several studies reported antimetastatic effects of *miR-503* through dampening the expression of various targets such as *WEE1* [121], *PRMT1* [122], and *ARHGEF19* [123]. Decelerated cellular growth, inducement of apoptosis, and sensitization to chemotherapy were all events associated with *miR-503* gain of function and were collateral to its modulation of its determined targets viz. *eukaryotic translation initiation factor 4E* (*EIF4E*) [124] and *insulin-like growth factor 1 receptor* (*IGF-1R*) [125].

6.6. miR-101. *miR-101* has been a confirmed tumor suppressor and recurrently reported as a downregulated species in HCC. Marked clampdown of tumor growth has been linked to the modulation of the *HGF*/*c-MET* axis by *miR-101-3p* [126]. *miR-101* also attenuated the expression of the *zinc-finger protein 217* (*ZNF217*), a potent effector of malignant immortalization [127]. Further, vasculogenic mimicry, an insidious mechanism of *de novo* vasculogenesis by which cancer resists angiogenic arrest, was undermined by *miR-101* mimics, which sabotaged *TGF-β* and *SDF1* signaling in cancer-associated fibroblasts and impaired *VE-cadherin* expression [128]. Similar to *miR-503*, *miR-101-3p* also targeted *WEE1*, which was shown to sensitize Huh7 and PLC5 to radiotherapy, an effect that is partially abrogated in HCC by the lncRNA *nuclear-enriched abundant transcripts 1* and *2* (*NEAT1* and *NEAT2*) [129]. On top of that, *miR-101* subverted the *TGF-β1*-instigated build-up of extracellular matrix (ECM), reversing hepatic fibrosis, and blunted the levels of phosphorylated *PI3K*, *mTOR*, and *Akt* [130]. As with other epigenetic modulators, *miR-101* has been tried as a part of several combinatorial regimens. Synergy was reported with liposomal doxorubicin [131] and the lncRNA *LINC00052*, which promoted the expression of the 3p arm of *miR-101* that restricted the expression of *SRY-related HMG-box gene 9* (*SOX9*) [132].

As is evident in Figure 2 and Table 4, different miRNAs have common targets and inevitably a single target can be regulated by more than one miRNA, which creates an elaborate regulatory network and sometimes complicate the utilization of miRNAs for diagnostic and therapeutic purposes.

6.7. Long Noncoding RNAs. Another major class of nonprotein-coding RNAs that is central to HCC and which is gaining significant attention as of late is long noncoding RNAs

Table 4: MicroRNAs (miRNAs) with disease-modifying effects in HCC. The table shows the direction of microRNA expression associated with the therapeutic effects, the regulated targets, and the observed effects in HCC.

MicroRNA	Expression changes associated with therapeutic effects	Effect	Targets (and the direction of their therapeutic regulation)	Reference
let-7c	Upregulation	Induction of apoptosis and inhibition of proliferation	*LIN28B*, *ARID3B*, *Bcl-xL*, and *c-Myc* (downregulation)	[227]
miR-663b	Upregulation	Suppression of tumor proliferation and invasiveness	*GAB2* (downregulation)	[228]
miR26a	Upregulation	Growth inhibition, migration, invasion, colony formation; initiation of hepatoselective apoptosis. Enhancement of chemosensitivity	*CCND2*, *IL-6*, and *PIK3C2α* (downregulation)	[229, 230]
miR-122			*ADAM17*, *CCNG1*, *ADAM10*, and *Bcl-w* (downregulation)	
miR-621	Upregulation	Amelioration of tumor radiosensitivity	*SETDB1* (downregulation)	[231]
miR-299-5p	Downregulation	Suppression of proliferation, migration, and invasion; initiation of apoptosis	*SIAH1* (upregulation)	[232]
miR-577	Upregulation	Inhibition of EMT and metastasis	*HOXA1* (downregulation)	[233]
miR-501-3p	Upregulation	Inhibition of proliferation, EMT, migration, and invasion	*LIN7A* (Downregulation)	[234]
miR-378a	Upregulation	Inhibition of proliferation and enhancement of sensitivity to sorafenib-based chemotherapies	*VEGFR2*, *PDGFRβ*, *MMP-2*, and *c-Raf* (downregulation)	[235]
miR-204-5p	Upregulation	Inhibition of cellular proliferation and clonogenicity	SIX1 (downregulation)	[236]
miR-495	Upregulation	Inhibition of proliferation and invasion	*IGF1R* (downregulation)	[237]
miR-758-3p	Upregulation	Inhibition of proliferation, migration, and invasion	*MDM2* and *mTOR* (downregulation)	[238]
miR-30a-5p	Upregulation	Inhibition of proliferation and invasion	*FOXA1* (downregulation)	[239]
miR-196a	Downregulation	Induction of apoptosis	*FOXO1* (upregulation)	[240]
miR-30a	Upregulation	Induction of apoptosis	*KRAS* (downregulation)	[241]
miR-326	Upregulation	Induction of apoptosis and inhibition of proliferation and invasion	*LASP1* (downregulation)	[242]
miR-708	Upregulation	Inhibition of proliferation, migration, and invasion	*SMAD3* (downregulation)	[243]
miR-296-5p	Upregulation	Inhibition of proliferation, migration, and invasion	*AKT2* (downregulation)	[244]
miR-24-1	Upregulation	Downregulation of c-Myc at the protein level and suppression of its O-GlcNAcylation; reduction of metastatic potential	*OGT* (downregulation)	[245]
miR-203a-3p *miR-548aa* *miR-376b-3p* *miR-548v* *miR-4510*	Upregulation	Inhibition of proliferation	*GPC3* (downregulation)	[246]
miR-211-5p	Upregulation	Inhibition of proliferation and apoptosis; enhancement of drug sensitivity	*ZEB2* (downregulation)	[247]
miR-138	Upregulation	Promotion of TRAIL-induced apoptosis	*ISG15* (downregulation)	[248]
miR-592	Upregulation	Inhibition of proliferation, migration, and invasion	*IGF-1R* (downregulation)	[249]
miR-365	Upregulation	Initiation of apoptosis	*Bcl-2* (downregulation)	[250]

Table 4: Continued.

MicroRNA	Expression changes associated with therapeutic effects	Effect	Targets (and the direction of their therapeutic regulation)	Reference
miR-217	Upregulation	Suppression of proliferation, migration, and invasion; initiation apoptosis	*MTDH* (downregulation)	[251]
miR-199a-5p	Upregulation	Decreased cell viability and colony formation; cell cycle arrest	*CLTC* (downregulation)	[119]
miR-185	Upregulation	Inhibition of proliferation; G0/G1 arrest; promotion of apoptosis	*RHEB*, *RICTOR*, and *AKT1* (downregulation)	[252]
miR-503	Upregulation	Repression of proliferation and sensitization to anticancer drugs	*EIF4E* (downregulation)	[124]
		Inhibition of invasion and migration; repression of EMT	*PRMT1* (downregulation)	[122]
miR-377	Upregulation	Suppression of proliferation and induction of apoptosis	*Bcl-xL* (downregulation)	[253]
miR-199a-3p	Upregulation	Growth inhibition and induction of apoptosis	*ZHX1* and *PUMA* (upregulation) and *Bcl-2* (downregulation)	[166]
miR-22	Upregulation	Inhibition of proliferation, migration, and invasion	*CD147* (downregulation)	[254]
miR-101	Downregulation	Repression of TGF-β and CD206 in M2 cells; inhibition of macrophage-driven HCC	*DUSP1* (upregulation)	[255]
	Upregulation	Suppression of proliferation, colony formation, EMT, and angiogenesis as well as VM. Inhibition of intrahepatic and distant metastases. Synergized with doxorubicin or fluorouracil to induce apoptosis	*TGF-βR1*, *Smad2*, *SDF1*, *VE-cadherin*, *EZH2*, *COX2*, *STMN1*, and *ROCK2* (downregulation)	[128, 256, 257]
miR-3178	Upregulation	Inhibition of proliferation, G1 arrest, and promotion of apoptosis	*EGR3* (downregulation)	[258]
LNA-antimiR-214	Upregulation	Reduction in fibrosis	*miR-214* (downregulation)	[259]
miR-190a	Upregulation	Suppression of migration and invasion	*treRNA* (downregulation)	[260]
miR-491	Upregulation	Lowering of cancer stem cell-like properties; inhibition of extracellular signal-regulated kinases	*GIT-1* (downregulation)	[261]
miR-497	Upregulation	Inhibition of colony formation and tumor growth	*IGF-1R* (downregulation)	[262]
miR-663	Downregulation	Inhibition of proliferation and promotion of apoptosis	*TGFβ1* (upregulation)	[263]
miR-20a	Upregulation	Promotion of apoptosis; inhibition of proliferation, invasion, and migration	*CCND1* (downregulation)	[264]
miR-148a	Upregulation	Suppression of tumor growth and malignancy. Promotion of differentiated phenotype	*IKKα* (downregulation)	[110]
miR-381	Upregulation	Inhibition of proliferation, colony formation, invasion, and induction of G0/G1 arrest	*LRH-1* (downregulation)	[265]
miR-27a-3p	Upregulation	Inhibition of EMT, metastasis, and VM	*VE-cadherin* (downregulation)	[266]
miR-26b-5p	Upregulation	Suppression of Twist1-induced EMT	*SMAD1* (downregulation)	[267]
miR-30a-5p	Upregulation	Inhibition of proliferation, colony formation, and induction of apoptosis	*MTDH* (downregulation)	[268]
miR-33a-3p	Upregulation	Suppression of cellular growth and migration/invasion	*PBX3* (downregulation)	[269]
miR-145	Upregulation	Inhibition of activation and proliferation of hepatic stellate cells	*ZEB2* (downregulation)	[270]
miR-1258	Upregulation	Inhibition of proliferation, G0/G1 arrest, and induction of apoptosis	*CKS1B* (downregulation)	[271]

Table 4: Continued.

MicroRNA	Expression changes associated with therapeutic effects	Effect	Targets (and the direction of their therapeutic regulation)	Reference
miR-1299	Upregulation	G0/G1 arrest and inhibition of proliferation	*CDK6* (downregulation)	[272]
miR-200a	Upregulation	Inhibition of EMT and decreased mitochondrial metabolism	*CXCL1* (downregulation)	[273]
miR-486-5p	Upregulation	Repression of proliferation, cellular viability, migration, and clonogenicity	*IGF-1R*, *mTOR*, *STAT3*, and *c-Myc* (downregulation)	[274]
miR-199a-5p	Upregulation	Inhibition of proliferation, migration/invasion, and synergized with chemotherapeutics	*E2F3* (downregulation)	[275]
miR-1285-3p	Upregulation	Inhibition of proliferation	*JUN* (downregulation)	[276]
miR-449a	Upregulation	Inhibition of motility and pulmonary metastasis; increase of epithelial markers and reduction of mesenchymal markers; reduction of Snail nuclear accumulation	*FOS* and *Met* (downregulation)	[277]
miR-302b	Upregulation	Sensitization to 5-FU	*MCL-1* and *DPYD* (downregulation)	[278]
miR-143	Downregulation	Inhibition of proliferation due to a G0/G1 arrest; induction of apoptosis	*TLR2*, *NF-κB*, *MMP-2*, *MMP-9*, *CD44*, *MMP14*, *integrin β1*, and *integrin β4* (downregulation)	[279]
miR-324-5p	Upregulation	Subduing invasiveness and metastatic capacity; downregulation of MMP2 and MMP9	*ETS1* and *SP1* (downregulation)	[279]
miR-26b	Upregulation	Inhibition of proliferation, invasion, and migration	*EphA2* (downregulation)	[280]
miR-449	Upregulation	Suppression of DNA replication, mitotic entry, and cellular proliferation	*SIRT1* and *SREBP-1c* (downregulation)	[281]
miR-221	Downregulation	Lowering of proliferation and clonogenicity; inhibition of migration/invasion; induction of G1 arrest and apoptosis	*BMF*, *BBC3*, and *ANGPTL2* (downregulation)	[282]
miR-206	Upregulation	Cell cycle arrest and inhibition of proliferation, invasion, and migration. Induction of apoptosis	*Notch3*, *HES1*, *Bcl-2*, and *MMP-9* (downregulation) and *p57*, *Bax*, and *cleaved caspase 3* (upregulation)	[283, 284]
miR-148a	Upregulation	Repression of EMT and pulmonary metastasis; increase of epithelial markers; reduction of mesenchymal markers	*Met* (downregulation)	[111]
miR-152	Upregulation	Inhibition of proliferation, cellular motility, and promotion of apoptosis	*TNFRF6B* (downregulation)	[285]
miR-99a	Upregulation	Inhibition of proliferation	*Ago2* (downregulation)	[286]
Anti- miR-197	Upregulation	Inhibition of migration and invasion; upregulation of CD82	*miR-197* (downregulation)	[287]
miR-26b	Upregulation	Sensitization of cells to doxorubicin-induced apoptosis	*TAK1* and *TAB3* (downregulation)	[288]
let-7a	Upregulation	Inhibition of local invasion and migration	*KRAS*, *HRAS*, and *NRAS* (downregulation)	[289]
miR-126-3p	Upregulation	Inhibition of migration and invasion; suppression of capillary tube formation; reduction of tumor volume and microvessel density	*LRP6* and *PIK3R2* (downregulation)	[104]
miR-302c	Upregulation	Attenuation of HUVECs motility; upregulation of VE-cadherin; downregulation of β-catenin, FSP1, and α-SMA; growth inhibition in cocultures	*MTDH* (downregulation)	[290]

Table 4: Continued.

MicroRNA	Expression changes associated with therapeutic effects	Effect	Targets (and the direction of their therapeutic regulation)	Reference
miR-148b	Upregulation	Inhibition of proliferation, metastasis and angiogenesis. Improvement of chemosensitivity	*NRP1* (downregulation)	[291]
miR-1188	Upregulation	Inhibition of proliferation, migration, invasion, and promotion of apoptosis	*Bcl-2* and *Sp1* (downregulation)	[292]
miR-126	Upregulation	Inhibition of proliferation, cell cycle arrest, and induction of apoptosis	*SOX2* (downregulation)	[105]

ADAM10: ADAM metallopeptidase domain 10; *ADAM17*: ADAM metallopeptidase domain 17; *Ago2*: Argonaute 2; *AKT1*: AKT serine/threonine kinase 1; *AKT2*: AKT serine/threonine kinase 2; *ANGPTL2*: Angiopoietin-like 2; *ARID3B*: AT-rich interaction domain 3B; *BAD*: Bcl-2-associated agonist of cell death; *BAX*: Bcl-2-associated X; *BBC3*: Bcl-2 binding component 3; *Bcl-2*: B-cell lymphoma 2 apoptosis regulator; *Bcl-w*: Bcl-2-like protein 2; *Bcl-xL*: B-cell lymphoma extra large; *BMF*: Bcl-2 modifying factor; *CCND1*: Cyclin D1; *CCND2*: Cyclin D2; *CCNG1*: Cyclin G1; *CD133*: CD133 antigen (prominin-1); *CD147*: Cluster of differentiation 147 (Basigin); *CDK6*: cyclin-dependent kinase 6; *CKS1B*: CDC28 protein kinase regulatory subunit 1B; *CLTC*: clathrin heavy chain; *c-Myc*: Myc protooncogene; BHLH transcription factor; *COX2*: cytochrome C oxidase subunit II; *c-Raf*: Raf-1 protooncogene, serine/threonine kinase; *CXCL1*: C-X-C motif chemokine ligand 1; *DPYD*: Dihydropyrimidine dehydrogenase; *DUSP1*: Dual specificity phosphatase 1; *E2F3*: E2F transcription factor 3; *EGR3*: EGR3 early growth response 3; *EIF4E*: eukaryotic translation initiation factor 4E; *EphA2*: Ephrin receptor A2; *ETS1*: ETS protooncogene 1; *EZH2*: enhancer of zeste 2 polycomb repressive complex 2 subunit; *FOS*: Fos protooncogene, AP-1 transcription factor subunit; *FOXA1*: Forkhead box A1; *FOXO1*: Forkhead box O1; *GAB2*: GRB2-associated-binding protein 2; *GIT-1*: GIT ArfGAP 1; *GPC3*: Glypican 3; *HES1*: Hairy and enhancer of split-1; *HOXA1*: Homeobox A1; *IGF1R*: insulin-like growth factor 1 receptor; *IKKα*: Inhibitor of κB kinase *α*; *IL-6*: interleukin-6; *ISG15:* interferon-stimulated gene 15; *JUN*: Jun protooncogene; AP-1 transcription factor subunit; *LASP1*: LIM and SH3 protein 1; *LIN28B*: Lin-28 homolog B; *LIN7A*: Lin-7 homolog A, crumbs cell polarity complex component; *LRH-1*: liver receptor homolog-1; *LRP6*: low-density lipoprotein receptor-related protein 6; *MCL-1*: MCL1 apoptosis regulator; *MDM2*: MDM2 protooncogene; *MET*: MET protooncogene, receptor tyrosine kinase; *MMP-2*: matrix metalloproteinase-2; *MMP-9*: matrix metalloproteinase-9, p57; *MTDH*: metadherin; *mTOR*: mammalian target of rapamycin; *NRP1*: Neuropilin-1; *OGT*: O-GlcNAc transferase; *OTUD7B*: OTU deubiquitinase 7B; *PBX3*: Pre-B-cell leukemia homeobox 3; *PDGFRβ*: Platelet-derived growth factor receptor beta; *PIK3C2α*: phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha; *PIK3R2*: phosphoinositide-3-kinase regulatory subunit 2; *PRMT1*: protein arginine methyltransferase 1; *PUMA*: p53 upregulated modulator of apoptosis; *RHEB*: Ras homolog, mTORC1 binding; *RICTOR*: RPTOR-independent companion of MTOR, complex 2; *ROCK2*: Rho-associated coiled-coil containing protein kinase 2; *SDF1*: Stromal cell-derived factor 1; *SETDB1*: SET domain bifurcated histone lysine methyltransferase 1; *SIAH1*: Siah E3 ubiquitin protein ligase 1; *SIRT1*: Sirtuin 1; *SIX1*: SIX homeobox 1; *SMAD1*: SMAD family member 1; *SMAD2*: SMAD family member 2; *SMAD3*: SMAD family member 3; *SOX2*: sex-determining region Y-box 2; *SP1*: Transcription factor Sp1 (specificity protein 1); *SREBP-1c*: Sterol regulatory element binding protein-1c; *STAT3*: signal transducer and activator of transcription 3; *STMN1*: Stathmin 1; *TAB3*: TGF beta-activated kinase binding protein 3; *TAK1*: Transforming growth factor beta-activated kinase 1; *TGFβ1*: Transforming growth factor beta 1; *TGF-βR1*: Transforming growth factor beta receptor 1; *TNFRF6B*: Tumor necrosis factor receptor super family 6B; *TNIP2*: TNFAIP3 interacting protein 2; *VEGFR*: vascular endothelial growth factor receptor; *ZEB2*: Zinc finger E-box binding homeobox 2; *ZHX1*: Zinc fingers and homeobox 1.

(lncRNAs). lncRNAs are a bit longer than miRNAs with a transcript length of more than 200 nucleotides [133]. lncRNAs have been extensively researched for their role in HCC pathogenesis and their therapeutic potential. As will be exposited shortly, a number of lncRNAs function by what is known as miRNA sponges, which basically involves buffering the action of miRNAs on their target mRNAs.

Given the comprehensive nature of this review, only some of the most recent reports involving lncRNA in HCC are discussed below. However, detailed information about earlier reports can be found in the following reviews: [134–136]. Additionally, the following bibliographic data [134–214] afford an extensive exposition of the most recent HCC lncRNA-oriented work. Beside the compendious run-through below, Table 5 affords an encyclopedic overview of the lncRNAs studied in these resources which were not discussed in the text for practical reasons.

6.8. GAS8-AS1. It was recently reported that both the *GAS8* gene and its resident lncRNA, *GAS8-AS1*, act as tumor suppressors and manifest a significantly low expression in HCC tissues, which correlated with poor prognosis [157]. *GAS8-AS1* was curiously found to mediate the transcription of *GAS8*. It was essential in maintaining chromatin in an uncondensed state by recruiting the H3K4 methyltransferase *MLL1* and its accessory protein *WD-40 repeat protein 5* (*WDR5*). This leads to the potentiation of RNA polymerase II and enhanced transcription of *GAS8*. The above molecular events suppressed oncogenesis and impeded HCC development.

6.9. FENDRR. FOXF1 adjacent noncoding developmental regulatory RNA (*FENDRR*), another lncRNA that was found to be downregulated in HCC, was recently advocated as a potential therapeutic approach to arrest HCC progression and discourage metastasis. Ectopic expression of *FENDRR* was reported to check malignant growths *in vitro* and *in vivo*, as well as repressing HCC migration and invasion. This was purported to occur via epigenetic regulation of *glypican-3* (*GPC3*). Through interacting with the *GPC3* promoter and subsequently leading to its methylation, *FENDRR* functions to silence *GPC3*, counteracting the latter's oncogenic effects [168].

6.10. CASC2c. Cancer susceptibility candidate 2c (*CASC2c*) is one of three lncRNA transcripts produced by the alternative splicing of *cancer susceptibility 2* (*CASC2*). Inherently silenced in HCC, the overexpression of *CASC2c* resulted in the suppression of proliferation of HCC cells, while inducing apoptosis. These effects coincided with lowered phosphorylated *extracellular signal-regulated kinase 1/2* (*p-ERK1/2*) and *β-catenin* levels [201].

6.11. miR503HG. miR503HG, the host gene of *miR-503* (see above), has been found to be significantly downregulated in HCC [141]. This silencing was closely related to survival rates and duration until tumor recurrence and is thus conjectured to be a prognostic biomarker. The gain of function abrogated the invasion and metastasis of HCC cells. *miR503HG* was also found to promote the degradation of the *heterogeneous nuclear ribonucleoprotein A2/B1* (*HNRNPA2B1*) by ubiquitination and subsequent proteasomal degradation, which consequently led to the destabilization of *p52* and *p65* transcripts and ultimately suppressed *NF-κB* signaling in HCC. Given their innate interplay and their common effect on HCC cells, *miR503HG* and its resident microRNA (*miR-503*) could cooperatively function to stymie migration of HCC cells.

6.12. LINC00467. LINC00467, another lncRNA that was found to be downregulated in HCC, has been studied as a potential therapeutic target thanks to its role as an antagomir for *miR-9-5a*, which targets *peroxisome proliferator-activated receptor alpha* (*PPARA*) for silencing [140]. *LINC00467* ectopically expressed in HCC cells conduced to antiproliferative effects and, like *miR503HG*, checked migration and invasion. The authors propose a pivotal implication of the *LINC00467*/*miR-9-5p*/*PPARA* loop in the initiation and progression of HCC.

6.13. Linc-GALH and UC001kfo. Contrary to the above-mentioned lncRNAs, which are downregulated in HCC and which are considered tumor suppressors, other lncRNAs are oncogenic, with anomalously high expression in HCC. *Linc-GALH* and *UC001kfo* were recently reported to be upregulated in HCC. *Linc-GALH* was surmised to regulate methylation of *Gankyrin* and hence its expression [190]. Mechanistically, this was proposed to occur via deubiquitinating DNMT1. This promoted migration and invasion in HCC cells and was rescinded in silencing experiments. Increased expression of *UC001kfo* correlated with tumoral macrovascular invasion (MVI) and TNM staging of HCC, with higher levels predisposing to poorer prognoses [179]. *UC001fko* boosted tumor proliferation and EMT, presumably through targeting *alpha-smooth muscle actin* (*α-SMA*). The authors indicate the potential of *UC001kfo* to serve as a prognostic marker as well as a target for therapy.

6.14. LINC00346. LINC00346 was shown to be aberrantly upregulated in HCC [139]. *LINC00346* enhanced the expression of *WD Repeat Domain 18* (*WDR18*) by virtue of competitively binding to *miR-542-3p*, a downregulated tumor suppressor in HCC cells. This sponging effect leads to the activation of the *Wnt*/*β-catenin* pathway. As such, *LINC00346* could be a viable target in HCC therapy, where its inhibition is presumed to unmask the anticancer effects of *miR-542-p*.

6.15. LINC00978. Both tumor tissues and serum samples from HCC patients manifested an exaggerated expression of *LINC00978* [69]. Serum levels of this lncRNA could even distinguish between HCC patients and patients with hepatitis or cirrhosis. *LINC00978* was reported to promote cellular proliferation, migration, and invasion, wherein its knockdown arrested the cell cycle and encouraged apoptosis. The authors unveiled the mechanistic basis of such effects to involve binding of *LINC00978* to *EZH2*, leading to its buildup at the promoter regions of *E-cadherin* and *p21* genes, which leads to these genes becoming silenced subsequent of *EZH2*-mediated H27K3 trimethylation. The validity of this regulatory circuit was confirmed by the abrogation of

Table 5: Dysregulated long noncoding RNAs (lncRNAs) in HCC. Long noncoding RNAs are shown with the trend of dysregulation associated with HCC. As is evident, the majority of dysregulated lncRNAs follow an upward tendency. Also evident is the involvement of lncRNA-mediated miRNA sponging in producing the oncogenic molecular phenotypes.

lncRNA	Expression in HCC	Effect of dysregulation	Ref.
91H	Upregulated	Promoting tumor growth and metastasis; upregulation of *IGF2*, H3K4me3, and H3K27me3 at the P3 and P4 promoters	[208]
AC006262.5	Upregulated	Inhibition of *miR-7855-5p* and upregulation of *BPY2C*	[200]
AC092171.4	Upregulated	Inhibition of *miR-1271* and upregulation of *GRB2*	[182]
ANCR	Upregulated	Enhanced proliferation and EMT; upregulation of *HNRNPA1* through *miR-140-3p* sponging	[151]
ANRIL	Upregulated	Inhibition of *miR-384* and upregulation of *STAT3*	[214]
ASMTL-AS1	Upregulated	Upregulation of *NLK* and activation of *YAP* signaling via *miR-342-3p* sponging	[293]
CASC2c	Downregulated	Activation of *ERK1/2* and *Wnt/β-catenin* signaling	[201]
CASC15	Upregulated	Activation of *Wnt/β-catenin* signaling via upregulation of *SOX4*	[196]
CRNDE	Upregulated	Inhibition of the *Hippo* pathway	[210]
CTBP1-AS2	Upregulated	Sponging of *miR-195-5p* and enhancing *CEP55* expression	[198]
DANCR	Upregulated	Enhanced cell proliferation, colony formation, and autophagy; upregulation of *ATG7* and suppression of *miR-222-3p*	[188]
DDX11-AS1	Upregulated	Inhibition of *LATS2* expression via *EZH2* and *DNMT1*	[203, 204, 207]
DUXAP8	Upregulated	Enhanced cell proliferation and EMT; *miR-422a* sponging and upregulation of *PDK2*	[147]
FENDRR	Downregulated	Downregulation of *GPC3*	[168]
FOXD2-AS1	Upregulated	*miR-206* sponging and enhanced *MAP3K1* signaling	[174]
FOXD3-AS1	Upregulated	*miR-335* sponging and upregulation of *RICTOR*	[159]
GAS8-AS1	Downregulated	Attenuated *GAS8* transcription RNA polymerase II activity	[157]
H19	Upregulated	Amelioration of resistance to sorafenib and upregulation of *miR-675*	[177]
HAND2-AS1	Downregulated	Enhanced proliferation; upregulation of *miR-300* and inhibition of *SOCS5*	[155]
HBVPTPAP	Upregulated	Activation of *JAK/STAT* signaling	[186]
HCG18	Upregulated	Upregulation of *CENPM* via sponging of *miR-214-3p*	[180]
HEIH	Downregulated	Suppression of cell proliferation and metastasis; upregulation of *miR-199a-3p*	[169]
HLNC1	Upregulated	Destabilization of *USP49*	[183]
HOTAIR	Upregulated	Downregulation of *c-Met* and *miR-34a*	[178, 184]
HOXA11-AS	Upregulated	Downregulation of *miR-506-3p* and *Slug*	[191]
KCNQ1OT1	Upregulated	Upregulation of *ACER3* via sponging of *miR-146a-5p*; enhanced sorafenib resistance and *PD-L1*-mediated immune escape via *miR-506* sponging	[197, 205]
LALR1	Upregulated	Anaplasia and distant metastases; upregulation of *SNORD72*	[154]
LEF1-AS1	Upregulated	Enhancement of tumor growth and chemoresistance; inhibition of *miR-10a-5p* and upregulation of *MSI*, *CDCA7*, and *EZH2*	[162, 194]
LINC00160	Upregulated	Inhibition of miR-132 and elevated levels of PIK3R3	[144]
LINC00174	Upregulated	Enhanced proliferation and metastasis and decreased apoptosis; sponging of *miR-320* and upregulation of *S100A10*	[152]
LINC00467	Downregulated	Sponging of miR-9-5a and consequent upregulation of *PPARA*	[140]
	Upregulated	Posttranscriptional inhibition of *NR4A3*	[153]
LINC00662	Upregulated		[294]

Table 5: Continued.

lncRNA	Expression in HCC	Effect of dysregulation	Ref.
		Genome-wide hypomethylation; modulation of *MAT1A*/*SAM* and *AHCY*/*SAH* interactions, leading to reduced *SAM* and increased *SAH*	
LINC00668	Upregulated	Promoting cell proliferation and EMT; sponging of *miR-532-5p* and consequent upregulation of *YY1*	[161]
LINC00978	Upregulated	Inhibition of *p21* and *E-cadherin* via *EZH2*-mediated silencing	[211]
LINC01224	Upregulated	Inhibition of *miR-330-5p* and consequent upregulation of *CHEK1*	[212]
LINC01278	Upregulated	Promoting metastasis; inhibition of *miR-1258*	[164]
LINC01296	Upregulated	Positive regulation of the *miR-26a*/*PTEN* axis	[137]
LINC01419	Upregulated	Histone methylation of the *RECK* promoter via *EZH2*	[173]
Linc-GALH	Downregulated	Upregulation of *Gankyrin*	[190]
lncARSR	Upregulated	Reduction of *YAP1* phosphorylation and activation of *IRS2*/*AKT* signaling	[156]
lncRNA-POIR	Upregulated	Enhanced EMT and sorafenib resistance; sponging of *miR-182-5p*	[202]
MALAT1	Upregulated	Tumor progression and doxorubicin resistance; *miR-3129-5p* sponging, upregulation of *β-catenin*	[199, 209]
MFI2-AS1	Upregulated	Improved proliferation and metastasis; sponging of *miR-134* and upregulation of *FOXM1*	[142]
MINCR	Upregulated	Enhanced proliferation and inhibition of apoptosis; downregulation of *miRNA-107*	[150]
miR503HG	Downregulated	Enhanced invasion and metastasis; activation of *NF-κB* signaling	[141]
MSC-AS1	Upregulated	Promoting cell proliferation and colony formation; suppression of *PGK1*	[172]
MT1JP	Downregulated	Repression of tumor growth; decreased *AKT* expression	[170]
NEAT1	Upregulated	Upregulation of *WEE1* through *miR-101-3p* sponging; inhibition of *miR-129-5p*	[129, 138]
OIP5-AS1	Upregulated	Promoting cell proliferation, migration and angiogenesis. Inhibition of apoptosis; inhibition of the *miR-26a-3p* and *miR-3163*	[163, 171]
OTUD6B-AS1	Upregulated	Enhanced proliferation and colony formation; sponging of *miR-664b-3p*	[181]
PICSAR	Upregulated	Enhanced proliferation and colony formation; sponging of *miR-588*	[189]
RHPN1-AS1	Upregulated	Promoting proliferation, migration and invasion; suppression of *miR-485-5p*	[165]
RUNX1-IT1	Downregulated	Desponging of *miR-632* and activation of *WNT*/*β-catenin* pathway	[148]
RUSC1-AS1	Upregulated	Enhanced proliferation and reduced apoptosis; *miR-7-5p* sponging and upregulation of *NOTCH3*	[185]
SLC2A1-AS1	Downregulated	Suppression of glycolysis in HCC cells; downregulation of *GLUT1*	[158]
SNAI3-AS1	Upregulated	Promoting proliferation and metastasis; activation of *PEG10* sponging *miR-27-3p* and *miR-34a-5p*	[195]
SNHG1	Upregulated	Enhanced tumor progression and metastasis; sponging of *miR-377-3p*	[149]
SNHG5	Upregulated	Sponging of *miR-26a-5p* and upregulation of the downstream target, *RNF38*	[160]
SNHG14	Upregulated	Inhibition of *miR-656-3p*, promotion of migration and invasion	[176, 187]
SOX2OT	Upregulated	Promoting the Warburg effect and metastasis; upregulation of *PKM2* via *miR-122-5p* inhibition	[167]
SUMO1P3	Upregulated	Enhanced cell proliferation and lymph node metastasis; *miR-320a* sponging and activation of *Wnt*/*β-catenin* signaling	[146]
TCL6	Downregulated	Activation of PI3K/AKT signaling via upregulation of *miR-106a-5p*	[145]
TMPO-AS1	Upregulated	Promoting proliferation, migration, and invasion; *miR-329-3p* sponging	[166]
TUG1	Upregulated	Negative regulation of *miR-137* and *AKT2* and promoting EMT	[175]
UBE2R2-AS1	Upregulated	*miR-302b* sponging and upregulation of *EGFR*	[192]
UC001kfo	Upregulated	Enhanced proliferation, macrovascular invasion, and EMT; upregulation of *α-SMA*	[179]

Table 5: Continued.

lncRNA	Expression in HCC	Effect of dysregulation	Ref.
ZFAS1	Upregulated	Enhanced proliferation; *miR-193a-3p* suppression	[213]
ZFPM2-AS1	Upregulated	Enhanced proliferation, migration, and invasion; inhibition of *miR-139*	[193]
ZNF281	Upregulated	Promoting migration and invasion; downregulation of *miR-539*	[143]

ACER3: Alkaline Ceramidase 3; *AHCY*: Adenosylhomocysteinase; *AKT*: Protein kinase B; *ATG7*: Autophagy-related 7; *BPY2C*: Basic Charge Y-Linked 2C; *CDCA7*: Cell Division Cycle-Associated 7; *CENPM*: Centromere Protein M; *CEP55*: Centrosomal Protein 55; *CHEK1*: checkpoint kinase 1; *c-Met*: Tyrosine-protein kinase *Met*; *DNMT1*: DNA methyltransferase 1; *EGFR*: epidermal growth factor receptor; *ERK*: extracellular signal-regulated kinase; *EZH2*: enhancer of zeste homolog 2; *FOXM1*: Forkhead box protein M1; *GAS8*: growth arrest-specific 8; *GLUT1*: Glucose transporter 1; *GPC3*: Glypican 3; *GRB2*: growth factor receptor-bound protein 2; *HNRNPA1*: heterogeneous nuclear ribonucleoprotein A1; *IGF2*: insulin-like growth factor 2; *IRS2*: *insulin receptor substrate 2*; *JAK*: Janus kinase; *LATS2*: large tumor suppressor 2; *MAP3K1*: mitogen-activated protein kinase 1; *MAT1A*: Methionine Adenosyltransferase 1A; *MSI*: RNA-binding protein Musashi; *NF-κB*: nuclear factor kappa-light-chain-enhancer of activated B cells; *NLK*: Nemo-Like Kinase; *NOTCH3*: Notch Receptor 3; *NR4A3*: Nuclear Receptor Subfamily 4 Group A Member 3; *p21*: cyclin-dependent kinase inhibitor 1; *PDK2*: Pyruvate dehydrogenase kinase isoform 2; *PD-L1*: Programmed death-ligand 1; *PEG10*: Paternally Expressed 10; *PGK1*: Phosphoglycerate Kinase 1; *PI3K*: Phosphoinositide 3-kinase; *PIK3R3*: Phosphoinositide-3-Kinase Regulatory Subunit 3; *PKM2*: Pyruvate kinase muscle isozyme; *PPARA*: peroxisome proliferator-activated receptor alpha; *PTEN*: Phosphatase and tensin homolog; *RECK*: Reversion-inducing-cysteine-rich protein with kazal motifs; *RICTOR*: Rapamycin-insensitive companion of mammalian target of rapamycin; *RNF38*: Ring Finger Protein 38; *S100A10*: S100 Calcium Binding Protein A10; *SAH*: S-adenosyl homocysteine; *SAM*: S-adenosyl-L-methionine; *SNORD72*: Small Nucleolar RNA, C/D Box 72; *SOCS5*: Suppressor of cytokine signaling 5; *SOX4*: SRY-Box Transcription Factor 4; *STAT3*: signal transducer and activator of transcription 3; *USP49*: Ubiquitin-Specific Peptidase 49; *WEE1*: WEE1 G2 Checkpoint Kinase; *YAP*/*YAP1*: Yes-associated protein 1; *YY1*: Yin Yang 1; *α-SMA*: *alpha*-smooth muscle actin.

LINC00978 knockdown's inhibitory effects in *E-cadherin* and *p21* knockdowns.

6.16. NEAT1. Nuclear-enriched abundant transcript 1 (*NEAT1*) is another lncRNA that is upregulated in HCC [138]. Silencing of *NEAT1* compromised cell viability and was shown to be proapoptotic in HepG2 and Huh7 cells. Again, as with other lncRNA/miRNA-negative correlations, *NEAT1* exhibited an opposite trend of expression to *miR-129-5p* in HCC. Ectopic expression of *NEAT1* suppressed *miR-129-5p* via modulating the *valosin-containing protein* (*VCP*)/*IκB* axis to the overall result of encouraging cellular proliferation.

6.17. ANRIL, LINC01296, and LINC01224. Similarly, *antisense noncoding RNA in the INK4 locus* (*ANRIL*), *LINC01296*, and *LINC01224* were all overexpressed in HCC and mediated their oncogenic effects through inhibition of microRNA signaling axes. *ANRIL*'s prooncogenic effects were found to rely on its suppression of *miR-384*, which targets *signal transducer and activator of transcription 3* (*STAT3*) [214]. These correlations were observed both *in vitro* and *in vivo*. *LINC01296* regulated the *miR-26a*/*PTEN* axis, resulting in tumor progression also *in vitro* and *in vivo* [137]. Similarly, an upswing of *LINC01224* in HCC was correlated with a silenced *miR-330-5p* and a consequent upregulation of its target, *checkpoint kinase 1* (*CHEK1*) [212]. *LINC01224* knockdowns exhibited a concurrent downregulation of *CHEK1*, owing to its binding to and inhibition of *miR-330-5p*, leading to tumor regression.

6.18. ZFAS1. HCC tissues exhibited an increased level of *ZFAS1*, compared to neighboring normal tissues [69]. The proliferative capacity of the tumor was substantially compromised subsequent of *ZFAS1* silencing, and its overexpression had a gainful effect on tumor growth. The authors report that the tumor suppressor miRNA, *miR-193a-3p*, was elevated in *ZFAS1* knockdowns which, confirmed by luciferase reporter assay and correlation analysis, suggested that the prooncogenic role of *ZFAS1* relied on the suppression of *miR-193a-3p*.

6.19. CRNDE. The *colorectal neoplasia differentially expressed* (*CRNDE*) lncRNA has recently been proven to be yet another prooncogenic lncRNA in HCC [210]. Its overexpression was associated with an enhanced proliferative and migratory competence of HCC cells, not to mention an ameliorated resistance to chemotherapy. *CRNDE* was determined to inhibit the Hippo pathway and encourage the *EZH2*-, *SUV39H1*-, and *SUZ12*-mediated inhibition of tumor suppressor genes viz. *large tumor suppressor 2* (*LATS2*) and *CUGBP Elav-like family member 2* (*CELF2*).

6.20. MALAT1. MALAT1 is a notoriously tumorigenic lncRNA implicated in many cancers. Recently, Chang et al. [209] proposed exploiting a *MALAT1*/*Wnt* regulatory loop for therapeutic purposes in HCC. They reported that *MALAT1* knockdowns evidenced a suppression of canonical *Wnt* signaling and impaired tumorsphere formation, which was coincident with a decline in CD90+ and CD133+ cells, which consolidated the hypothesis that *MALAT1* plays a vital role in promoting stemness in HCC cells.

7. Future Perspective

Despite the thorough study of epigenetic modulators, their extension to the clinical setting stands far from realizable. Further research mindful of the efficacy versus long-term toxicity/of these alternative strategies should be advocated. Studies looking into the pharmacokinetics of these agents as well as others seeking efficient targeted delivery with minimal systemic side effects are warranted. Addressing the adaptability of these modes of treatment to the clinic can bring us a long way, especially with the dosing curtailment of the highly toxic agents afforded by the concomitant use of the suggested alternatives, which, in some instances, may completely replace current debilitating treatments. As was mentioned, various exploratory clinical studies were carried out, but these need to be seen through to subsequent trial phases and on larger populations. Fortunately, the possible risk posed by a preponderance of these modulators is not significant to impede but should embolden such undertakings.

In addition to the clinical application, endeavors oriented to further our understanding of the elaborate epigenome and its regulation remain imperative. New epigenetic mechanisms are still being discovered contemporarily and progress in the field could do with pursuing modulators of these and assessing their benefits over the already defined ones. For example, decreased crotonylation of histone lysines has been recently incriminated in the progression of HCC [215]. This discovery should prompt several spin-offs in which the enhancers of crotonylation are suggested and assessed for therapeutic utility. Several defined modulatory agents such as histone demethylases (specifically Jumonji lysine demethylases) and helicases (HELLS) [216] among others also remain underresearched in HCC and should thus constitute a future research direction in HCC therapeutics.

8. Conclusion

The modulation of the altered epigenome in HCC is a promising therapeutic strategy. Verified potency and tenability to formulation demands for maximal systemic effects render many of the hereinabove nominated agents an intriguing recourse that could be subsequently implemented in clinical settings as a standalone curative or a potentiating adjuvant. It would also remain of equal importance to examine if these modulators can act in parallel to attenuate metastasis. More importantly, validating the use of these modulators in the treatment of HCC with different etiologies will aid in paving the road for personalized medicine together with the advancements in the pharmacogenomics/pharmacogenetics field. This holistic approach is forecasted to lower the success barrier, at least in part, in the treatment of HCC.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Investigation of the miRNA and mRNA Coexpression Network and Their Prognostic Value in Hepatocellular Carcinoma

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Purpose. To identify pivotal differentially expressed miRNAs and genes and construct their regulatory network in hepatocellular carcinoma. *Methods*. mRNA (GSE101728) and microRNA (GSE108724) microarray datasets were obtained from the NCBI Gene Expression Omnibus (GEO) database. Then, we identified the differentially expressed miRNAs and mRNAs. Sequentially, transcription factor enrichment and gene ontology (GO) enrichment analysis for miRNA were performed. Target genes of these differential miRNAs were obtained using packages in *R* language (*R* package *multiMiR*). After that, downregulated miRNAs were matched with target mRNAs which were upregulated, while upregulated miRNAs were paired with downregulated target mRNA using scripts written in Perl. An miRNA-mRNA network was constructed and visualized in Cytoscape software. For miRNAs in the network, survival analysis was performed. And for genes in the network, we did gene ontology (GO) and KEGG pathway enrichment analysis. *Results*. A total of 35 miRNAs and 295 mRNAs were involved in the network. These differential genes were enriched in positive regulation of cell-cell adhesion, positive regulation of leukocyte cell-cell adhesion, and so on. Eight differentially expressed miRNAs were found to be associated with the OS of patients with HCC. Among which, miR-425 and miR-324 were upregulated while the other six, including miR-99a, miR-100, miR-125b, miR-145, miR-150, and miR-338, were downregulated. *Conclusion*. In conclusion, these results can provide a potential research direction for further studies about the mechanisms of how miRNA affects malignant behavior in hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. It is estimated that 840,000 new cases of HCC are acquired and at least 780,000 people die of HCC every year, and over half of the global incidence and mortality of HCC occur especially in Eastern Asian [1]. HCC has a high incidence (4.7% of new cancer cases) and the second highest cancer mortality rate (8.2% of cancer-related deaths) worldwide [2], and China accounts for 47% of the total number of HCC cases as well as HCC-related mortality [3]. The development of HCC is closely related to the infection of the hepatitis B virus (HBV) infection, followed by the hepatitis C virus infection (HCV), and related to aflatoxins, alcohol drinking, and so on [4, 5]. In China, HCC has the third highest cancer incidence and has become the second leading cause of cancer-related death, second only to lung cancer [6]. Unfortunately, there are many difficulties in the diagnosis and treatment of HCC, but the most frequent is the lack of methods for early diagnosis as well as the paucity of studies about the molecular mechanisms of tumor initiation and progression. Therefore, we designed the study to explore the molecular mechanisms of HCC carcinogenesis and progression, as well as new relevant molecular markers for early diagnosis.

Recently, noncoding RNA (ncRNA) drawn extensive concern to further illustrate the molecular mechanisms of HCC. MicroRNAs (miRNAs), families of small noncoding RNAs, had been reported that can serve as molecular markers for early diagnosis of a number of tumors [7, 8]. Many of miRNAs (e.g., miR-1247-3p [9] and miR-935 [10]) have been demonstrated to play a role in the progression of HCC by influencing proliferation, invasion, and metastasis of tumor cells as well as other malignant phenotypes.

MiRNAs can promote the degradation of mRNAs and inhibit their translation into proteins by binding to the 3′-untranslated region (3′-UTR) of target mRNAs [11]. Thus, studies on miRNAs were important for inquiring the molecular mechanisms of carcinogenesis and to seek novel biomarkers.

Microarray profiling is a kind of high-throughput technique that developed rapidly in recent years, which can be applied to detect differentially expressed miRNAs and genes in cancer and control samples [12]. To find new directions for research in miRNAs and genes, we analyzed miRNA and mRNA microarray datasets to identified differentially expressed miRNAs and genes and explored their potential relationships. Next, we identified the key miRNAs with survival analysis, network analyses, and functional enrichment.

2. Materials and Methods

2.1. Microarray Data Collection. The raw miRNA and mRNA sequencing data were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), which represents the largest public repository of microarray data. In this study, one gene expression profile (GSE101728) and one miRNA expression profile (GSE108724) were downloaded from the GEO.

The GSE101728 dataset was composed of seven pairs of HCC and matched adjacent tumor-free tissue sample mRNA expression profiles which were collected during the surgery from HCC patients admitted to the Zhongshan Hospital of Fudan University [13]. The GSE108724 dataset includes the profiling of the miRNA expression in seven pairs of HCC and matched adjacent tumor-free tissues from the same hospital and research team [13]. All the data were obtained in a raw status and normalized with Perl (Perl version 5.32.3) and *R* (version: 4.0.2).

2.2. Data Processing. The raw data was downloaded, and the probe ID was transferred to gene symbol or miRNA name. The data from different groups was classified into the normal group and tumor group with packages in *R* language (*R* package *limma*), which was also used to screen the differentially expressed miRNAs and mRNA between the tumor and normal tissues. In the same time, we also calculated the log fold change (logFC), *P* values, and adjusted *P* values (adj. *P* val). In addition, adj. *P* value<0.05 and |logFC| >2 were set as the standards of differentially expressed miRNA and mRNA selection. According to the above standards, 37 differentially expressed miRNAs (15 upregulated and 22 downregulated) and 745 differentially expressed mRNAs (30 upregulated and 441 downregulated) were screened.

2.3. Prediction of miRNA Target Genes. After extracted the differential miRNAs, packages in *R* language are used to predict the target genes (*R* package *multiMiR*). The packages were published in 2014 [14], but were updated in April 2020 recently. The packages were integration of fourteen databases for prediction of the miRNA target gene, including miRecords (http://c1.accurascience.com/miRecords), miRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php), TarBase (http://diana.imis.athena-innovation.gr/DianaTools/index.php), and miRDB (http://mirdb.org/). And the intersection of the results of the fourteen databases was taken as the final result of the target gene prediction.

2.4. miRNA-mRNA Interaction Network Construction. Investigation of the miRNA and mRNA coexpression network is beneficial for the exploration of the molecular mechanism of hepatocellular carcinoma. To construct the miRNA-mRNA network, including positive and negative relationships between mRNA and miRNA, we extracted 35 miRNAs with target genes from all the 37 differentially expressed miRNAs and 295 target mRNAs from 745 differentially expressed mRNAs. We matched upregulated miRNA with their downregulated target mRNA and downregulated miRNA with their upregulated mRNA. In result, 330 nodes and 481 edges are included in the network. In this study, the miRNA-mRNA network was constructed using a Perl program, followed by visualization using Cytoscape software (version 3.8.0; 64-bit; http://www.cytoscape.org/) [15]. In addition, we obtained a list of 568 oncogenes and 1217 tumor suppressor genes that have been identified based on previous reports. Research on oncogenes has been reported, and the specific list of oncogenes can be downloaded on the OncoGenomics (intOGen) platform (https://www.intogen.org/search) [16]. The list of tumor suppressor genes can be downloaded in the Tumor Suppressor Gene Database (https://bioinfo.uth.edu/TSGene/). We divided the mRNAs in the network into tumor-promoting genes and tumor suppressor genes according to the obtained gene classification data and distinguished by different colors in the network.

2.5. Functional and Pathway Enrichment Analysis. We ran a transcription factor enrichment analysis and a GO enrichment analysis for differential miRNA for the three GO domains: molecular functions (MF), biological processes (BP), and cellular components (CC). A KEGG pathway analysis and a GO enrichment analysis, including MF, BP, and CC, of the mRNAs were subsequently performed. All the enrichment analysis was performed using FunRich software (version 3.1.3; http://www.funrich.org). $P < 0.05$ was applied as the criterion.

2.6. Overall Survival (OS) Analysis of the miRNAs. The related clinical data of HCC patients were downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) database. A total of 368 HCC patients with complete available miRNA expression and follow-up datasets were involved in the overall survival analysis. Patients were divided into two groups according to the median value of the expression of miRNA that we want to study (high vs. low expression). Kaplan-Meier survival analysis was performed, and *P* value of 5-year survival rates was calculated and displayed. For all the analysis and plotting process, the *R* package *Survival* (https://cran.r-project.org/web/packages/survival/index.html) was used.

2.7. Analyzed the Expression Data Downloaded from the TCGA Database. In addition to relevant clinical data, we have also downloaded data on the miRNA expression in normal

tissues and liver cancer tissues in TCGA. The aforementioned methods are used to process the data, to screen out the miRNAs that are abnormally expressed in liver cancer tissues, and to perform relevant multifactor COX regression analysis and survival analysis for differential miRNAs. Based on miRNAs that have an impact on the prognosis, target genes are predicted, and an interactive network of miRNA and mRNA is constructed. For oncogenes and tumor suppressor genes in the network, we also conducted survival analysis and calculated the difference in five-year survival rates.

3. Results

A total of 745 differentially expressed genes were identified from GSE101728 and 37 miRNAs from GSE108724. The conditions of judgment for significant differences are the log fold change > 2 and the adjusted P value <0.05. There are 304 upregulated genes and 441 downregulated genes among these differential genes. For miRNA, 15 miRNAs are upregulated, and 22 are downregulated (Figure 1). Heatmap of miRNA and mRNA differentially expression clearly distinguished HCC tissues (posterior seven samples) from paired adjacent normal tissues (Figures 1(a) and 1(c)). In the heatmap, red represents high expression, and green represents low expression. The comparison of colors can show the difference in the expression of miRNA and mRNA in the two sets of samples. In the volcano plot, green and red dots indicated the down and upregulation of the miRNA and mRNA expression in tumor and normal tissues, respectively. In the volcano map of miRNA, you can see the expression distribution of miRNA. Since the fold change is set to 4 times, most miRNAs are considered to have no expression differences, and only a small part of miRNAs are included in subsequent studies (Figures 1(b) and 1(d)).

For the selected differential miRNAs, we performed GO and transcription factors (TF) enrichment analysis. TF and GO analysis are hints for the research direction. Through the GO analysis, we can find the GO classification items of the differential miRNAs and find out which function changes may be related to the differential miRNAs. The results of TF and GO enrichment analysis of the differential miRNAs are shown in Figure 2. The results of TF analysis for differential upregulated and downregulated miRNA are shown below (Figure 2(a)–(c)). In the result of transcription factor enrichment, the blue column indicates the proportion of miRNA enriched on the transcription factor to all differential miRNAs. The red bar represents the value of $-\log_{10}$ (P value), and the yellow bar represents the threshold of statistical difference ($-\log_{10}$ 0.05, $P = 0.05$). The red bar is longer than the yellow band, indicating a statistical difference ($P < 0.05$). GO analyses cover three domains and show the top 10 significant GO enrichments according to enrichment scores [$-\log_{10}$ (P value)] (Figures 2(d)–(f)).

In order to show the relationship between differential miRNAs and target genes more intuitively, we used Cytoscape software to achieve visualization. The miRNA-mRNA regulatory network was constructed that consists of 35 miRNAs and 295 target mRNAs. Since miRNAs generally negatively regulate target genes, 17 upregulated miRNAs were matched with 144 downregulated target mRNAs while 18 downregulated miRNAs with 151 target mRNAs (Figure 3). In the network, we use different colors to represent different expression levels and different functions of miRNA and mRNA.

We not only performed GO enrichment analysis for miRNAs but also GO and KEGG enrichment analysis for differential mRNAs. GO enrichment analysis and KEGG pathway analysis of differentially expressed genes in the miRNA-mRNA network are shown in Figure 4. Representation of the genes in the GO enrichment bubble plot and circle plot displayed the count distribution in BP, CC, and MF. Bubble color intensity indicates fold enrichment of GO terms overrepresented in that cluster of genes, and the size corresponds to the number of genes enriched (count). The GO analysis results revealed that the differentially expressed genes were significantly enriched in the terms "reproductive structure development," "reproductive system development," "positive regulation of cell adhesion," etc (Figures 4(a) and 4(b)). The result of the KEGG pathway analysis was shown in the same form. The genes were significantly enriched in "MicroRNAs in cancer," "PI3K-Akt signaling pathway," and "Focal adhesion." The results of the enrichment analysis suggest that the effect of differential genes enriched in the corresponding items on the corresponding phenotype of tumor cells can be studied. For example, the KEGG enrichment analysis results of SRC show that it is enriched in the item of cellular adhesion, so basic experiments can be used to verify whether SRC has an effect on the invasion and metastasis of liver cancer cells..

If the difference in the expression of miRNAs is significantly related to the survival of patients, it means that this miRNA is likely to have greater research value. Therefore, for differential miRNAs in the network, OS analysis was performed. The data including the expression of miRNAs, follow-up time, survival state, and survival time were from the TCGA miRNA-seq dataset. Survival curves were plotted, and differences between survival curves were estimated (Figure 5). As a result, nine survival curves are statistically different (P <0.05); among them, hsa-mir-125b was divided into hsa-mir-125b-1 and hsa-mir-125b-2 (Figures 5(c) and 5(d)).

In addition to the GEO database, we also conducted data mining on the TCGA database. Perform data processing on the data downloaded from the TCGA database screen out a total of 53 abnormally expressed miRNAs and calculate the P value. All the different miRNAs are displayed in the chart and sorted by P value (Table 1). We drew a heatmap and a volcano map to visually show the difference in the expression and distribution of 53 differential miRNAs in normal tissues and cancer tissues (Figures 6(a) and 6(b)). In order to study the impact of differential miRNAs on the prognosis of patients, we used multivariate COX regression analysis and survival analysis methods. Multivariate COX regression analysis showed that 8 miRNAs are related to the prognosis of patients ($P < 0.05$). At the same time, we also calculated the hazard ratio of each miRNA. A hazard ratio greater than 1 indicates a negative impact on the prognosis, and the hazard

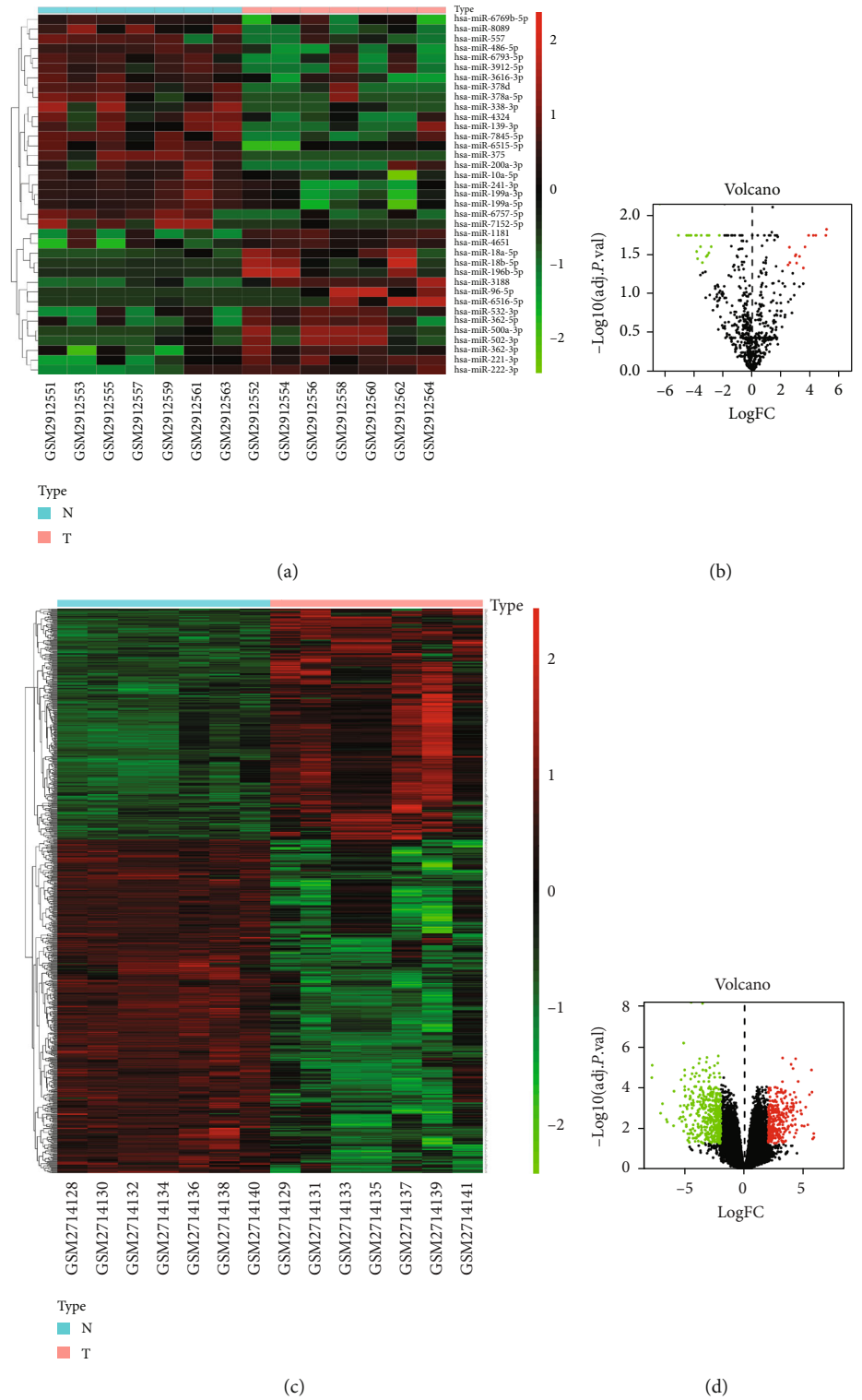


Figure 1: Heatmap and volcano plot of differentially expressed miRNAs and mRNAs. (a) Heatmap of differential miRNA microarray. (b) Volcano plot of differential miRNA microarray. (c) Heatmap of differential mRNA microarray. (d) Volcano plot of differential mRNA microarray.

ratio that is less than 1 indicates a benign effect on the prognosis (Figure 6(c)). Then, we drew survival curves for all the differential miRNAs based on the expression, the patient's survival time, and survival status and calculated the 5-year survival rate. The results showed that the 5-year survival rates of mir-9-1, mir-9-2, mir-9-3, mir-452, mir-514a-2, and mir-4800 were significantly different (Figures 6(d)–(i)).

To find more meaningful target-regulatory relationships and guide the next experiments, we constructed a network which only contains miRNAs with significant differences in

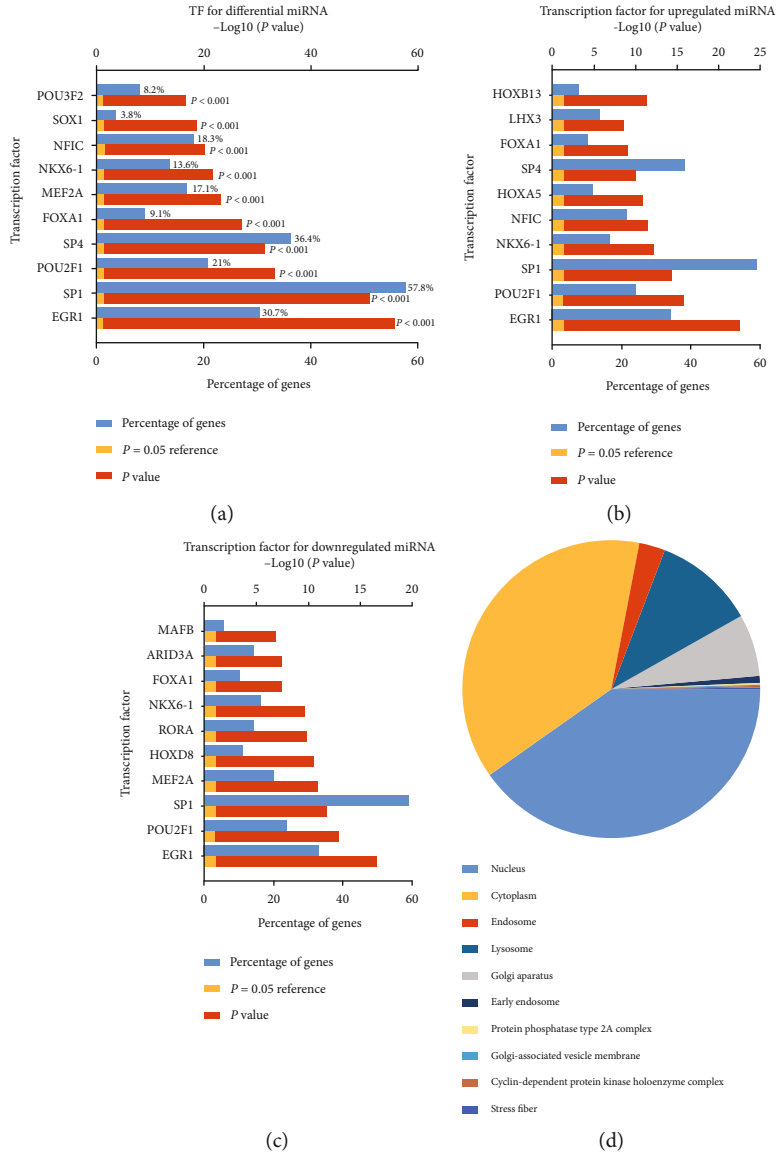


Figure 2: Continued.

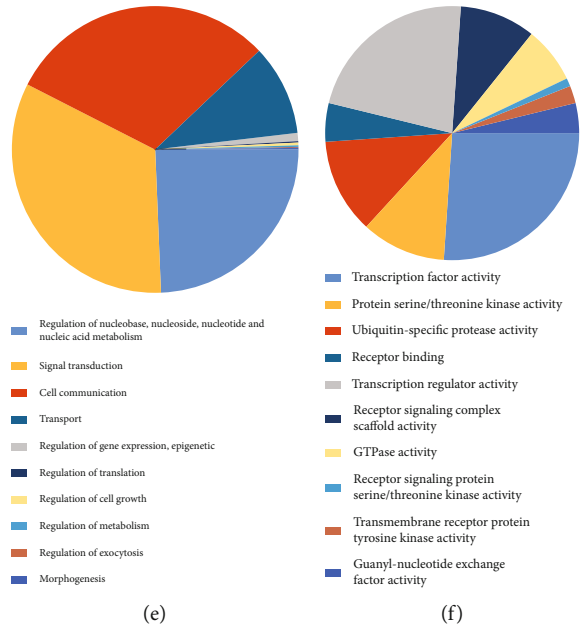


Figure 2: Enrichment analysis of transcription factors and GO terms for differential miRNA. (a) Transcription factor (TF) enrichment analysis for differential miRNA. (b) TF enrichment analysis for upregulated miRNA. (c) TF enrichment analysis for downregulated miRNA. (d) The enriched GO terms in the cellular component (CC). (e) The enriched GO terms in the biological process (BP). (f) The enriched GO terms in the molecular function (MF).

the survival curves (Figure 7). In the network, we also marked the oncogenes and tumor suppressor genes with different colors.

For oncogenes and tumor suppressor genes in the network, we conducted survival analysis and calculated the difference in five-year survival rates (Figure 8). We conducted survival analysis on the six tumor suppressor genes CDKN2B, DACT1, DUSP6, E2F3, IGFBP3, PLCE1, RASSF3, and THY1 and found that E2F3, IGFBP3, and RASSF3 genes had a significant impact on the 5-year survival rate of patients ($P < 0.05$). The 5-year survival rate of the oncogene GMPS high expression group was significantly lower than that of the low expression group.

4. Discussion

With a high mortality rate, substantial morbidity, and the increasing trend of the incidence rate of HCC worldwide, the pathogenesis, disease progression, and treatment of HCC are worthy of further study and exploration. Noncoding RNA has been an intensive research topic in molecular biology for several years and the focus of numerous studies [17, 18]. MiRNAs, families of small noncoding RNAs, played important roles in nearly all biological processes. Plenty of studies indicated that the abnormal expression of miRNAs may contribute to oncogenesis and the progression of HCC by inhibiting target genes through the degradation of their target mRNAs or by inhibiting translation [19, 20]. Thanks to the well-developed microarray technology, now it is easier to determine the expression levels of the miRNAs and mRNAs. We can identify differentially expressed miRNAs and genes between normal and tumor tissues and screen miRNAs that seem to play roles in tumor onset or progression. In addition, the result of microarray analysis can help give direction for future research.

In the study, a total of 35 miRNAs (17 upregulated and 18 downregulated) and 295 mRNAs (151 upregulated and 144 downregulated) were screened. The transcription factors for differentially expressed miRNA were enriched in EGR1, POU2F1, SP1, MEF2A, HOXD8, etc. GO enrichment analysis of these miRNAs showed that they are significantly enriched in "regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism", signal transduction, cell communication, and transport. The differentially expressed mRNA is enriched in the reproductive structure development, reproductive system development, and positive regulation of cell adhesion. For KEGG analysis of mRNAs, they are enriched in miRNAs in cancer, PI3K-Akt signaling pathway, focal adhesion, and Rap1 signaling pathway. Moreover, by constructing the miRNA-mRNA network and performing OS analysis, we identified miRNAs including miR-99a, miR-100, miR-125b, miR-145, miR-150, miR-324, miR-338, and miR-425, which were found to have an impact on the HCC survival rate. Thus, the network has been simplified.

miR-125b-5p is one of the downregulated miRNAs in tumor tissues of HCC patients compared to normal tissues. In the miRNA-mRNA network, it has the highest connectivity with target genes, which regulates 31 upregulated genes. Among these, there are 2 genes in the top 100 of total 979 upregulated genes including KIF18B and RBM24. Little research has been done on miR-125b in HCC, but some studies showed that miR-125b can affect the metastasis of gastric cancer cells and inhibit colorectal cancer proliferation [21, 22]. Recent research reported that KIF18B promotes hepatocellular carcinoma progression through activating the Wnt/β-catenin-signaling pathway [23], but the upstream

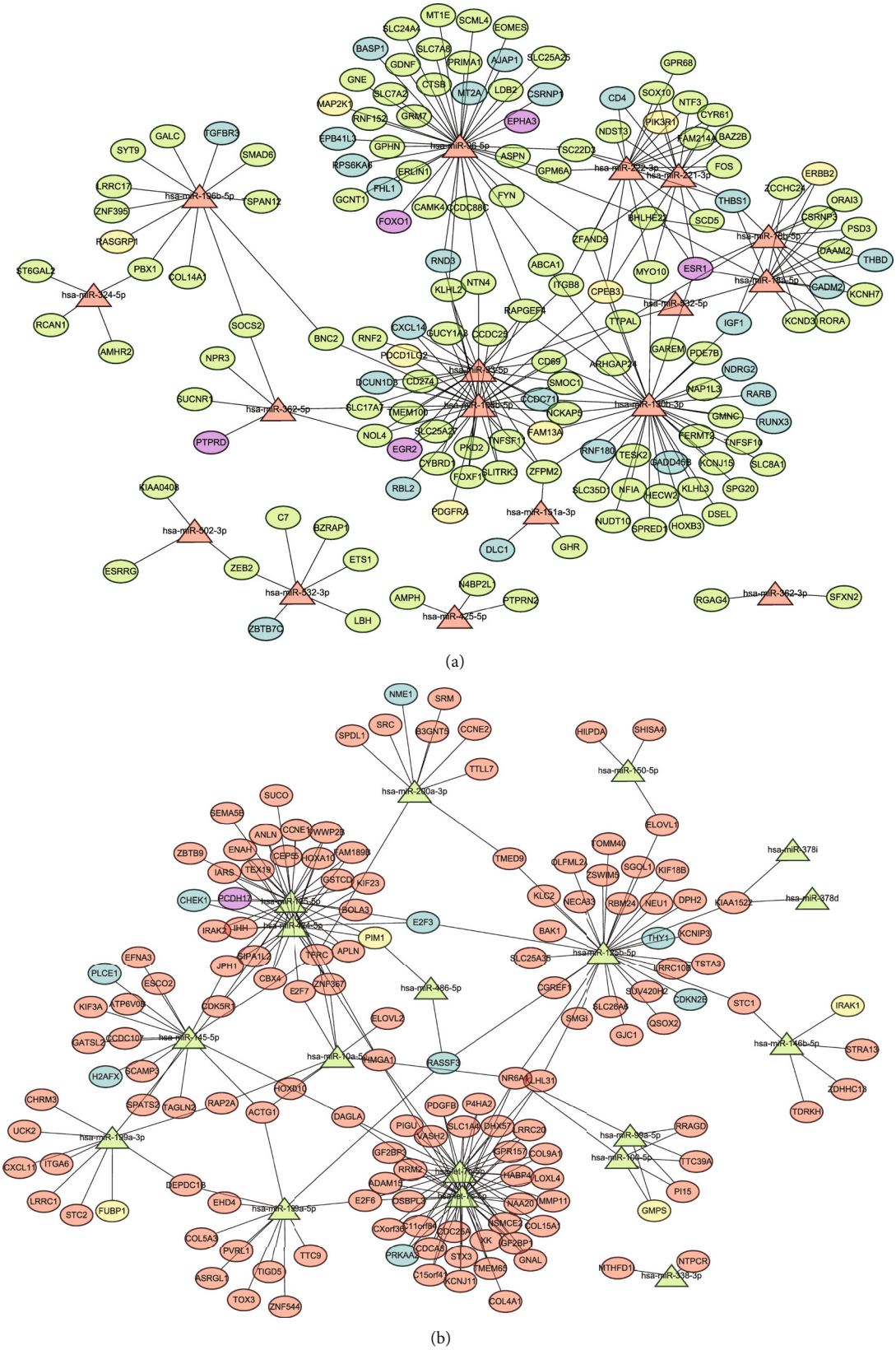
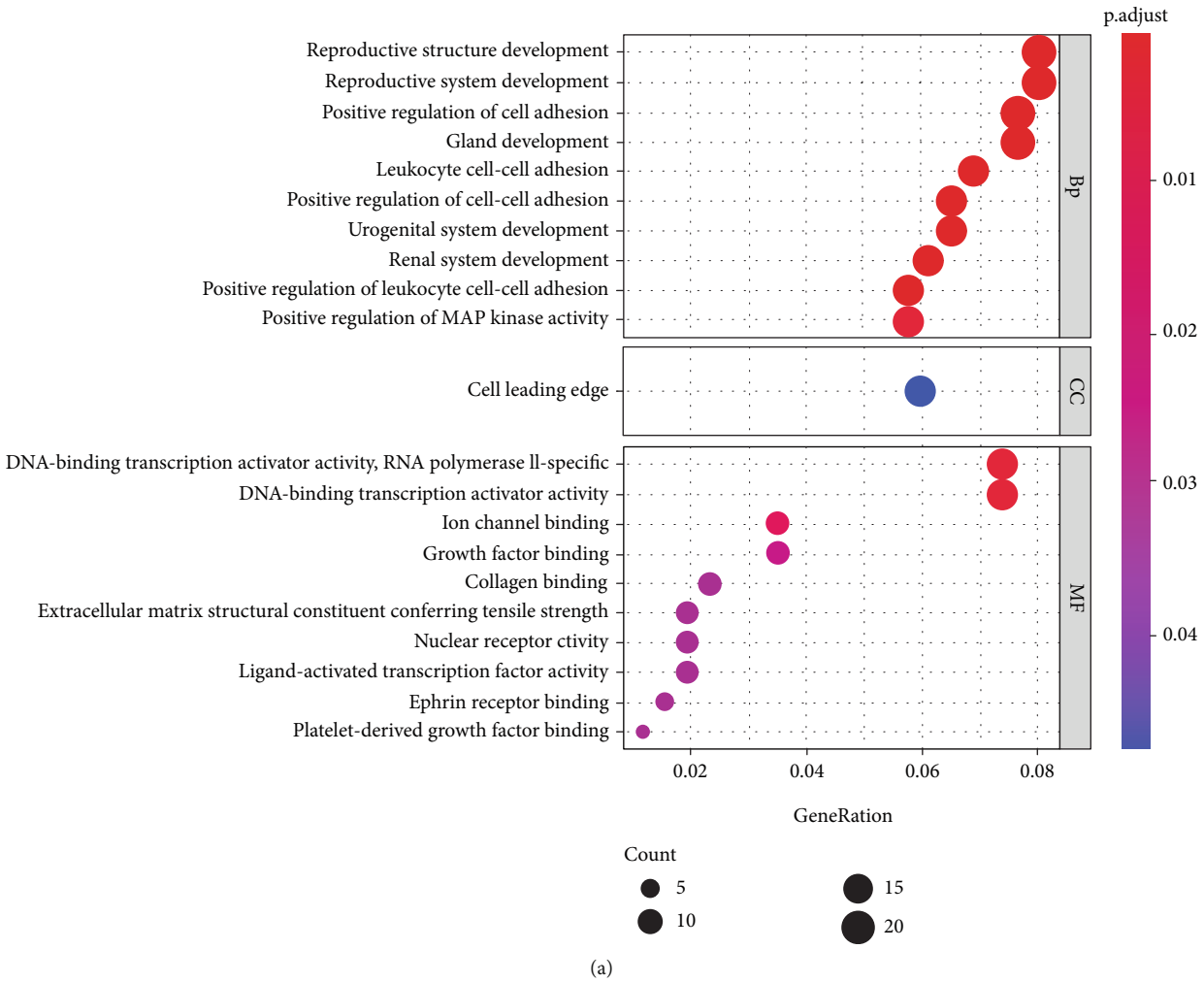


Figure 3: The miRNA-mRNA regulatory network consists of 35 miRNAs and 295 target mRNAs. (a) Regulatory network of upregulated miRNAs and downregulated mRNAs. (b) Regulatory network of downregulated miRNAs and upregulated mRNAs. Triangular nodes, miRNA; elliptical nodes, mRNA; green nodes, downregulation; pink nodes, upregulation; blue nodes, tumor suppressor gene; yellow nodes, oncogenes; purple nodes, at the same time in the list of oncogenes and tumor suppressor genes.



(a)

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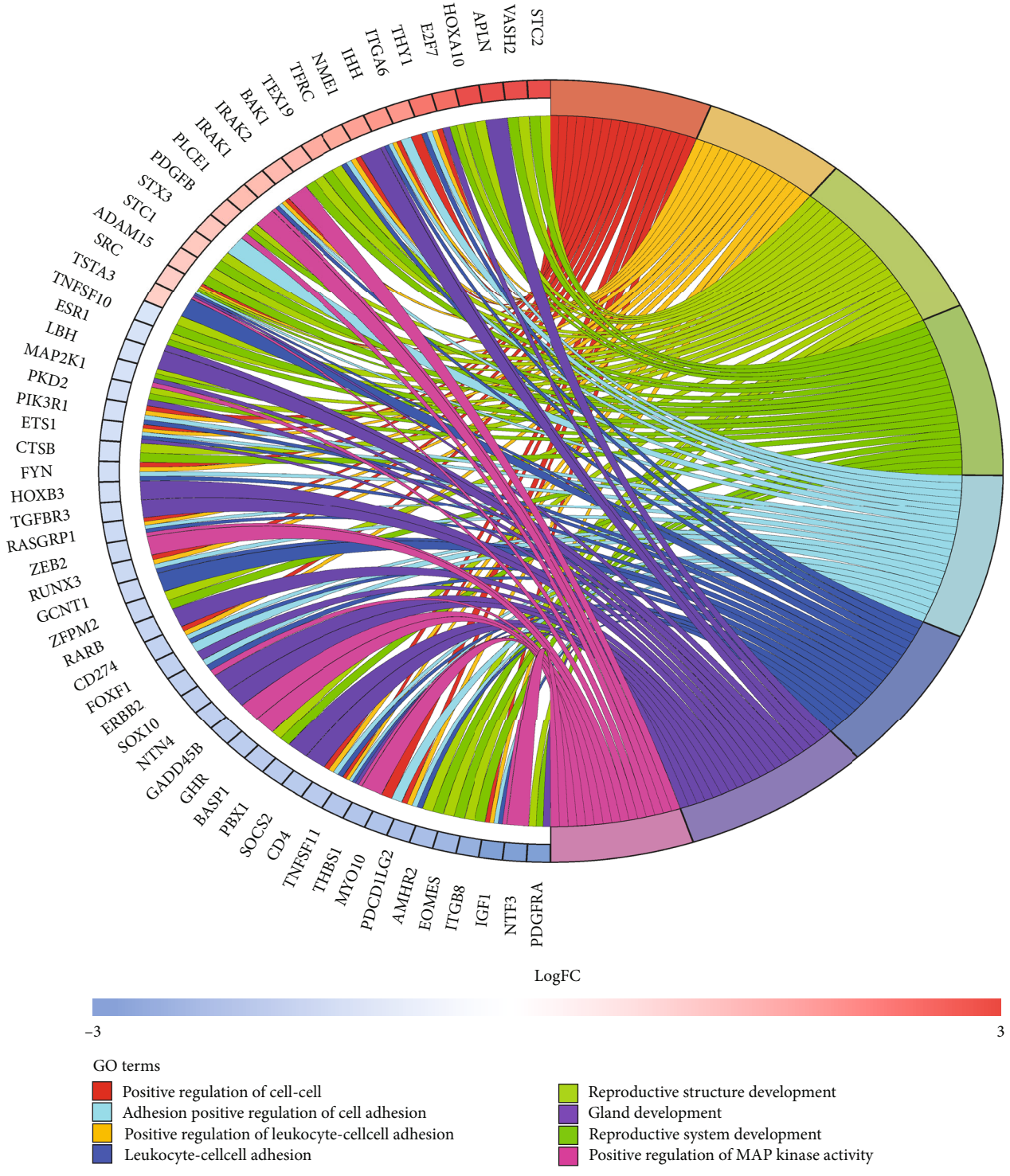

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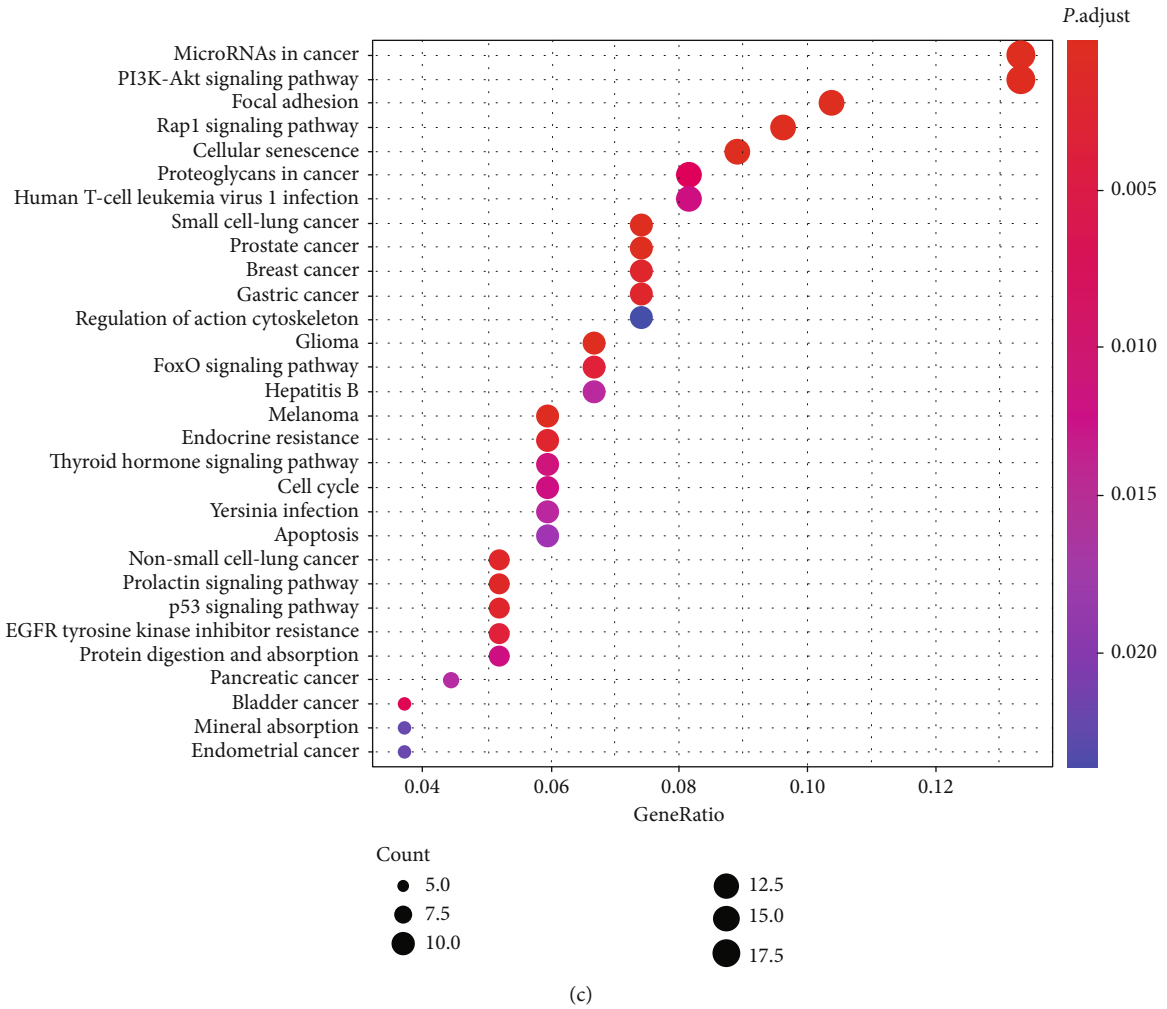

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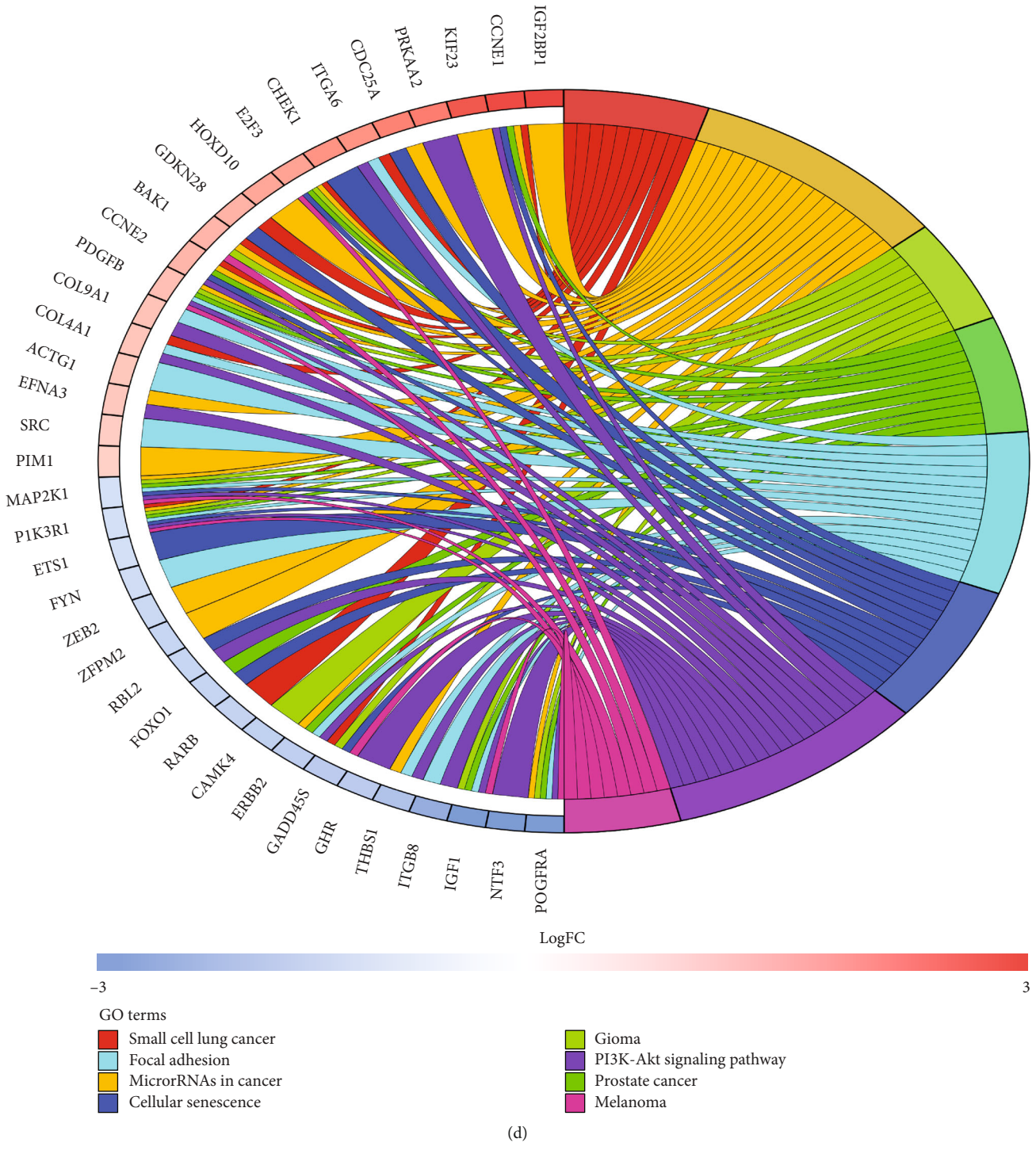
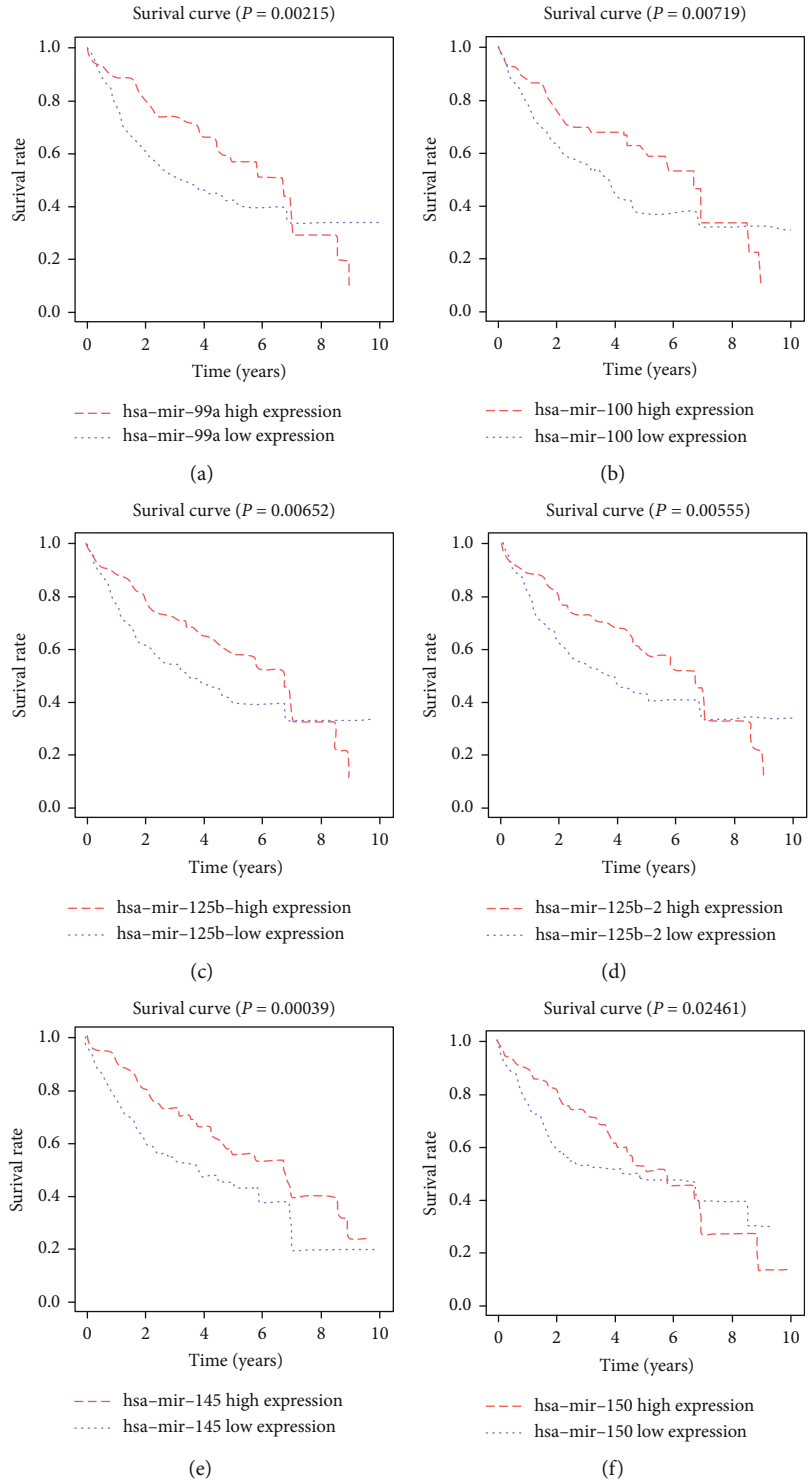


(d)

Figure 4: Bubble plot and circle plot of the GO/KEGG analysis of differentially expressed genes in the miRNA-mRNA network. (a) Bubble plot of the GO enrichment analysis. (b) Circle plot of the GO enrichment analysis. (c) Bubble plot of the KEGG enrichment analysis. (d) Circle plot of the KEGG enrichment analysis.

regulators of KIF18B are unknown. According to the network, the exact relationship between miR-125b and KIF18B needs to be verified through experiments.

Among target genes of miR-99a, peptidase inhibitor 15 (PI15) has been reported to act as a novel blood diagnostic marker for cholangiocarcinoma, and the plasma PI15 level in HCC patients was clearly higher than normal [24]. However, the physiological and pathological role of plasma PI15 is still unknown. Downregulation of GMP synthetases (GMPS), another target gene of miR-99a, can result in reduced cell viability as a p53 repression target in HCC [25]. Thus, the downregulation of miR-99a may inhibit tumor proliferation by upregulating GMPS, but it still requires experimental validation. Recent research showed



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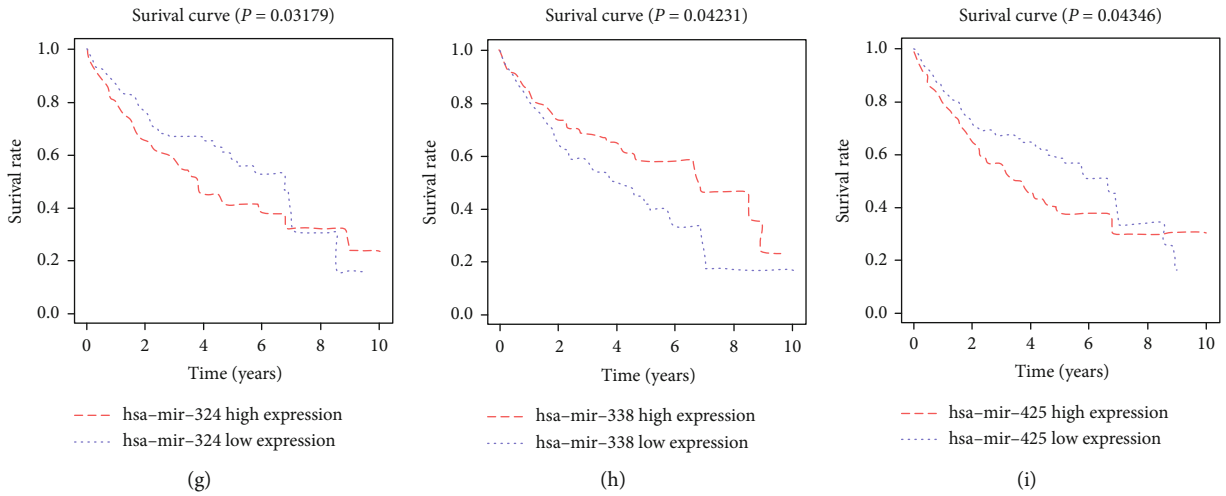
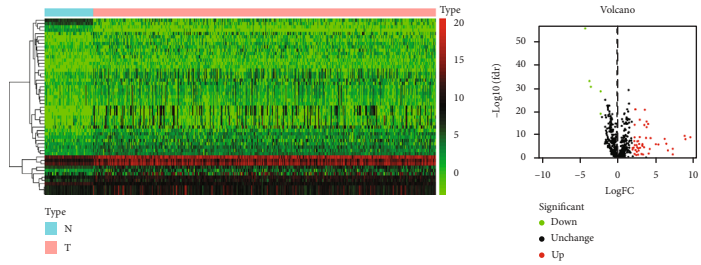


Figure 5: Overall survival analysis of high and low expression groups of (a) miR-99a, (b) miR-100, (c) miR-125b-1, (d) miR-125b-2, (e) miR-145, (f) miR-150, (g) miR-324, (h) miR-338, and (i) miR-425 in HCC.

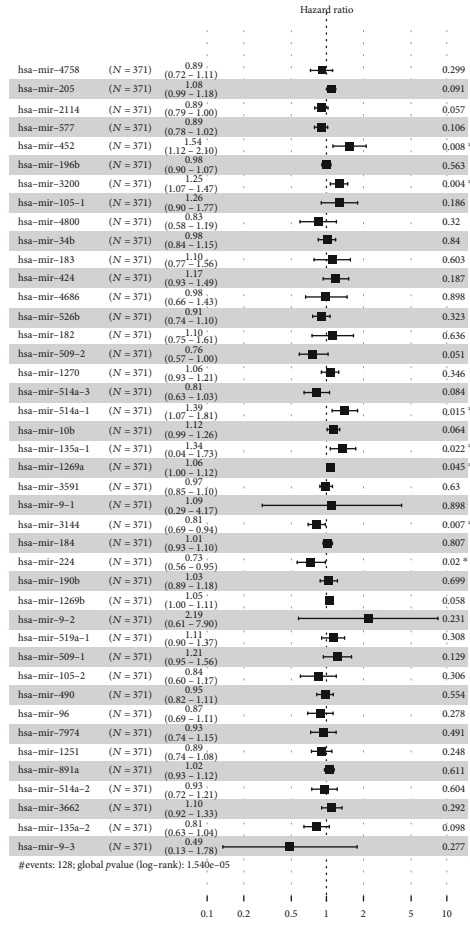
Table 1: Differentially expressed miRNAs in HCC in the TCGA database.

ID	LogFC	*P* value	ID	LogFC	*P* value
hsa-mir-4686	-4.1834	4.12E-59	hsa-mir-1251	5.1093	3.16E-07
hsa-mir-490	-3.6287	2.93E-36	hsa-mir-1269a	5.4367	4.14E-07
hsa-mir-1258	-3.4329	1.10E-33	hsa-mir-6783	2.3317	7.11E-07
hsa-mir-424	-2.1563	1.88E-31	hsa-mir-514a-3	2.5705	7.32E-07
hsa-mir-4746	2.4126	2.70E-23	hsa-mir-9-3	3.3295	8.76E-07
hsa-mir-10b	3.6625	4.32E-23	hsa-mir-9-2	3.3379	1.35E-06
hsa-mir-4800	-2.1605	3.04E-21	hsa-mir-7974	2.6364	6.24E-06
hsa-mir-224	2.9886	2.26E-18	hsa-mir-135a-2	3.5473	6.50E-06
hsa-mir-34c	3.9009	1.19E-17	hsa-mir-4758	2.1997	9.23E-06
hsa-mir-183	4.0866	2.11E-16	hsa-mir-135a-1	3.0362	1.09E-05
hsa-mir-452	2.3124	4.58E-16	hsa-mir-1270	2.0524	1.32E-05
sa-mir-182	3.5976	5.59E-16	hsa-mir-509-2	2.8006	2.43E-05
hsa-mir-96	3.9076	5.07E-15	hsa-mir-520a	7.3327	3.57E-05
hsa-mir-767	8.8785	4.72E-11	hsa-mir-184	4.3001	4.22E-05
hsa-mir-190b	2.9875	1.48E-10	hsa-mir-3591	2.0458	6.92E-05
hsa-mir-105-2	9.6441	1.75E-10	hsa-mir-514a-2	2.4440	0.00012
hsa-mir-3200	2.3078	1.89E-10	hsa-mir-526b	6.6936	0.00020
hsa-mir-34b	3.8027	4.14E-10	hsa-mir-509-3	2.8453	0.0002
hsa-mir-3144	4.4032	5.12E-10	hsa-mir-509-1	2.5959	0.0006
hsa-mir-891a	6.2658	1.04E-09	hsa-mir-205	4.1754	0.00467
hsa-mir-105-1	8.9992	1.32E-09	hsa-mir-1224	2.3070	0.00717
hsa-mir-3662	2.7909	1.03E-08	hsa-mir-2114	3.2124	0.00741
hsa-mir-508	2.2759	1.03E-08	hsa-mir-519a-1	7.3346	0.01289
hsa-mir-1254-1	2.6607	1.33E-08	hsa-mir-196b	3.4244	0.01445
hsa-mir-1269b	6.4907	1.62E-07	hsa-mir-541	2.7758	0.01715
hsa-mir-514a-1	2.9022	2.37E-07	hsa-mir-577	2.5328	0.02511
hsa-mir-9-1	3.3532	2.92E-07			

that nuclear receptor subfamily 6, group A, member 1 (NR6A1) regulates lipid metabolism of HepG2 cells, and the positive expression of NR6A1 is a novel marker of disease progression and aggressiveness in prostate cancer patients [26, 27]. Thus, the interaction between miR-99a and NR6A1 in tumor migration and invasion could be a direction for future research. In addition, a ceRNA network including miR-125b and miR-99a could also be constructed.



(a) (b)



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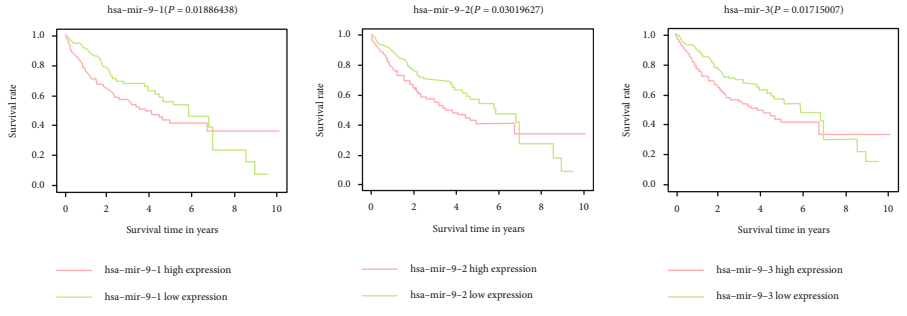

(d) (e) (f)

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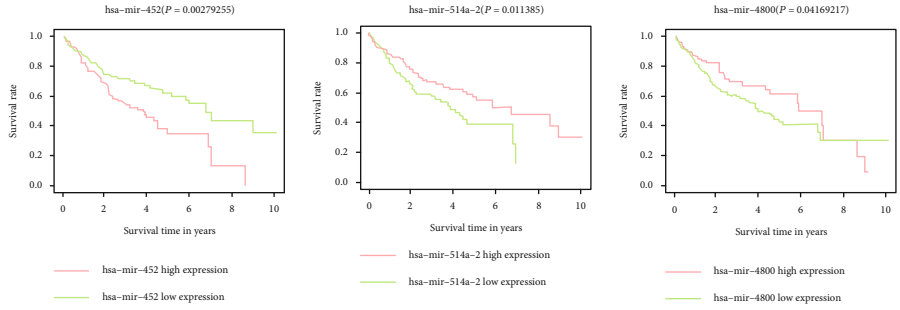
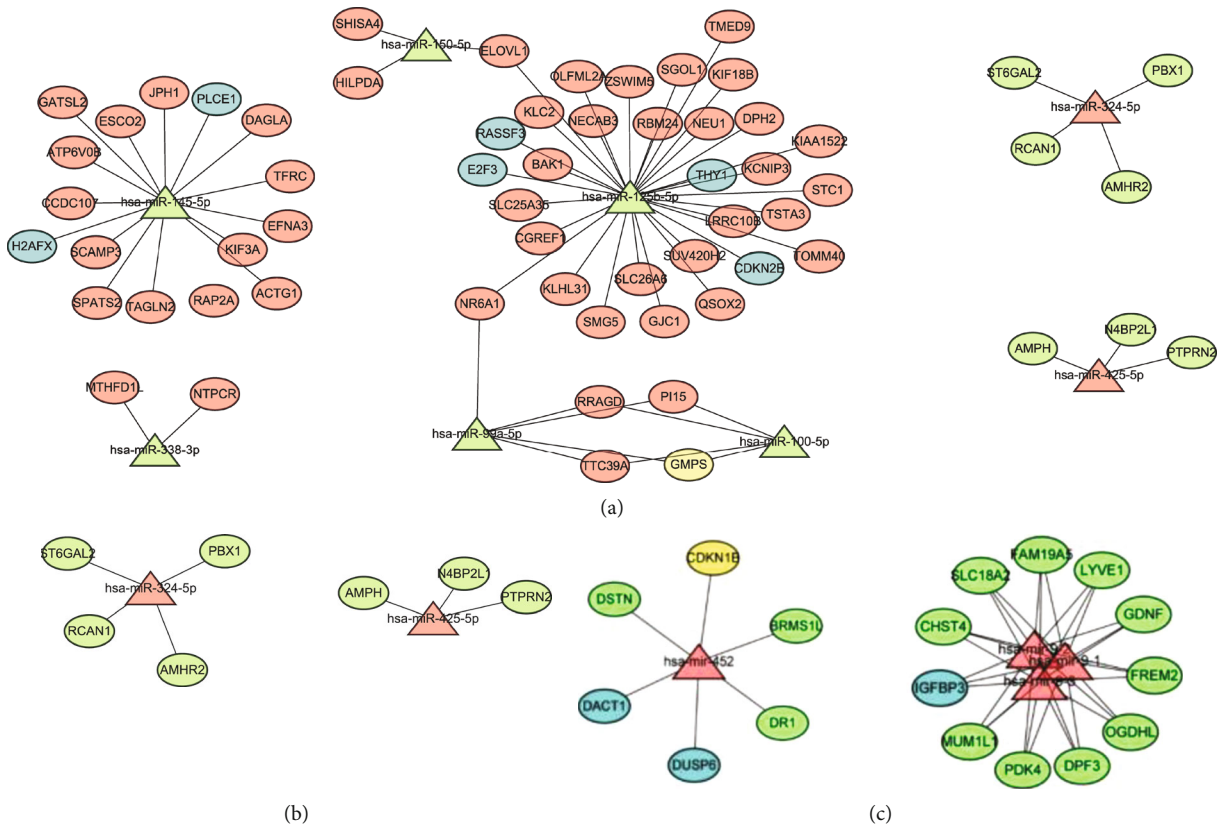


(g) (h) (i)

Figure 6: Analysis results of differential miRNA in TCGA. (a) Heatmap of differential miRNAs. (b) Volcano plot. (c) Multivariate COX regression analysis result. (d) Survival analysis curve of miR-9-1. (e) Survival analysis curve of miR-9-2. (f) Survival analysis curve of miR-9-3. (g) Survival analysis curve of miR-452. (h) Survival analysis curve of miR-514a-2. (i) Survival analysis curve of miR-4800.

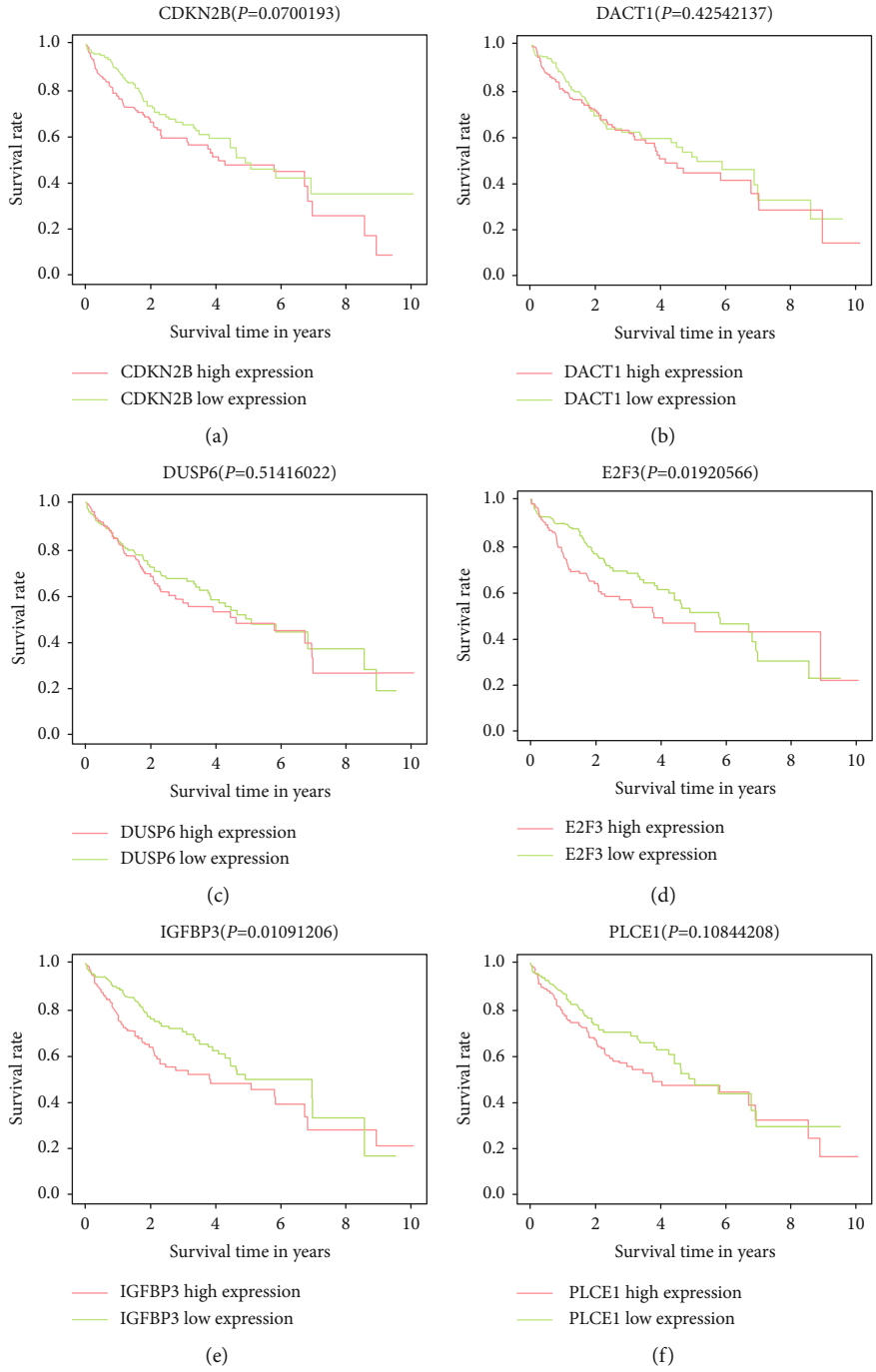


(a)

(b) (c)

Figure 7: The miRNA-mRNA regulatory network consists of miRNA with OS analysis difference and their target mRNA. (a) Network of downregulated miRNAs with OS analysis difference and their target mRNAs. (b) Network of upregulated miRNAs with OS analysis difference and their target mRNAs. (c) Network of upregulated miRNAs in the TCGA database with OS analysis difference and their target mRNAs in the legend. Triangular nodes, miRNAs; elliptical nodes, mRNAs; green nodes, downregulation; pink nodes, upregulation; blue nodes, tumor suppressor gene; yellow nodes, oncogenes.

For upregulated miRNA, miR-324-5P is correlated to patients' prognosis and has regulatory relationships with 4 genes. One research showed that miR-324-5p suppresses HCC cell invasion, but another study reported that lncRNA-85 promotes HCC cellular proliferation and migration by targeted binding and regulating miR-324-5p [28, 29]. Thus, the effect of miR-324-5P on tumor progression might be different via different mechanisms. Among target genes, the high expression of alpha-2,6-sialyltransferase 2 (ST6GAL2), one of the top 100 of all the 1236 downregulated genes, was demonstrated to promote tumorigenesis of follicular thyroid cancer via activating the Hippo signaling pathway [30], and the downregulation of ST6GAL2 is associated with improved patient survival in breast cancer [31], but the effects of ST6GAL2 have not been reported yet on the oncogenesis and the progression of HCC. Regulator of calcineurin 1(RCAN1) is broadly expressed in the liver, placenta, and other tissues. Overexpressed RCAN1, as a



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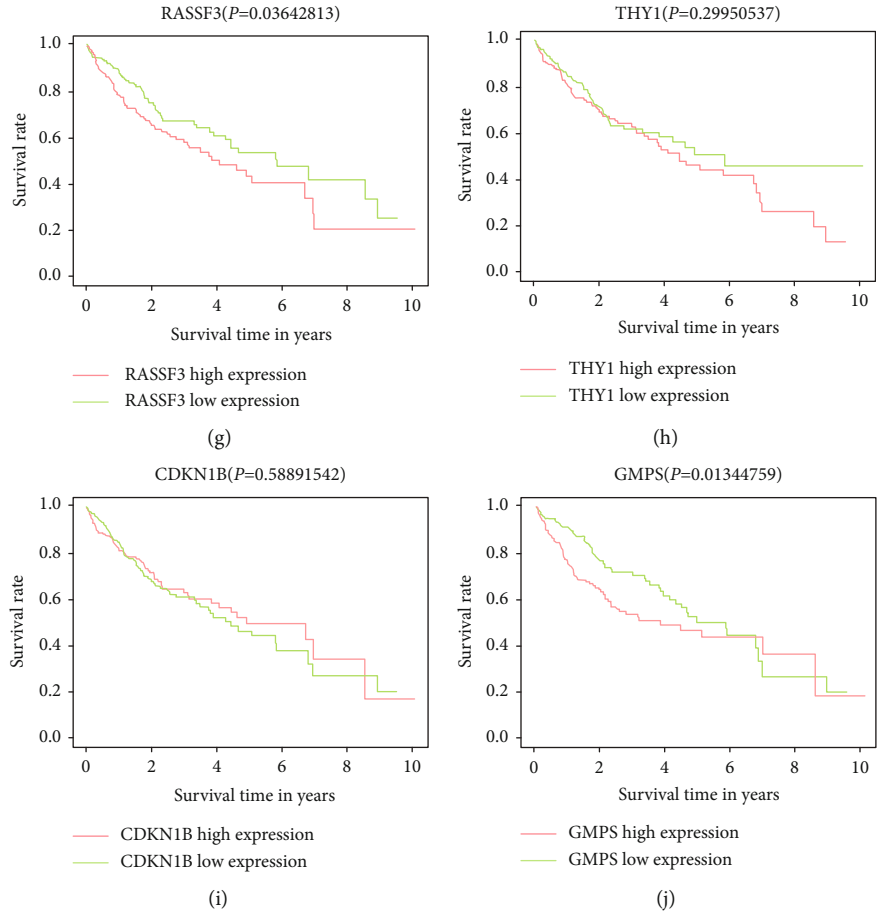

Figure 8: Survival analysis curves of oncogenes and tumor suppressor genes in the network. (a) CDKN2B. (b) DACT1. (c) DUSP6. (d) E2F3. (e) IGFBP3. (f) PLCE1. (g) RASSF3. (h) THY1. (i) CDKN1B. (j) GMP3.

potential target of miR-572, induced apoptosis of HCC cells and inhibited cell proliferation and invasion [32]. The regulatory relationship between miR-324-5P and RCAN1 has not been reported. Anti-Mullerian hormone receptor type 2 (AMHR2) encodes the receptor for the anti-Mullerian hormone (AMH) which results in male sex differentiation. PBX1 encodes a nuclear protein that belongs to the PBX homeobox family of transcriptional factors. The former two genes have a greater value of study than the latter.

MiR-425-5p promotes tumor progression in HCC [33], gastric cancer [34], breast cancer [35] and so on. Amphiphysin (AMPH) is a critical tumor suppressor that inhibits tumor progression in breast cancer [36], osteosarcoma [37], etc. Thus, the upregulation of miR-425 may negatively regulate AMPH to promote tumor progression. The relationship between miR-425 and AMPH has been reported that miR-425 regulates cell proliferation, migration, and apoptosis by targeting AMPH in non-small-cell lung cancer [38]. However, the relationship needs to be validated in HCC in further studies. There are few reports about the roles and mechanisms of NEDD 4 binding protein 2-like 1 (N4BP2L1) and protein tyrosine phosphatase receptor type N2 (PTPRN2) for their limited value.

Some miRNAs have tumor suppressor and carcinogenic effects, and the main mechanism is the binding of miRNA and target mRNA. The combination of miRNA and mRNA will cause a decrease in the expression level of target mRNA. Some studies have also found that miRNAs can bind to target mRNA to increase the translation of target mRNA [39]. Through these mechanisms, miRNAs can regulate the expression of many genes and play a similar role to oncogenes or tumor suppressor genes. In the interaction network, we have also marked out the oncogenes and tumor suppressor genes that have been identified. miRNAs and mRNAs transcribed from these genes have potential interaction and coexpression relationships. This can provide certain research directions for future research.

We identified 37 differentially expressed miRNAs and 745 mRNAs in tumor tissues of HCC patients compared to normal controls. 481 negatively regulatory pairs were used to construct a miRNA-mRNA interaction network including 35 miRNAs and 295 mRNAs. Then, we identified 8 miRNAs that are associated with the long-term survival rate and prognosis by using survival analysis. GO and KEGG pathway analyses revealed that the abnormal expression of miRNAs and genes may participate in the regulation of cell adhesion and then induce invasion and metastasis of tumor cells. There are limitations to the study. The sample size is relatively small, which may have an impact on the trustworthiness and credibility of the result of microarray analysis. In a

further study, we can get a large sample size of differentially expressed genes in miRNA or mRNA microarray datasets for screening differential miRNAs and mRNAs. Furthermore, the mechanisms of miRNA–mRNA regulatory relationship in the network require validation through laboratory-based experiments.

Data Availability

The mRNA (GSE101728) and microRNA (GSE108724) microarray datasets during the current study are available in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/).

Additional Points

In this paper, we analyzed miRNA and mRNA microarray datasets to identified differentially expressed miRNAs and genes and explored their potential relationships. And we identified the key miRNAs with survival analysis, network analyses, and functional enrichment. This study can provide a research direction for further study of molecular mechanism. We believe this manuscript is valuable for all the researchers who are interested in. This study includes not only the research about tumor-related miRNAs and genes but also the clinical research about differential miRNAs.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Hao Zhang analyzed the data and wrote the manuscript. Xi Chen participated in the manuscript writing. Yufeng Yuan designed the research. All authors read and approved the final manuscript.

Acknowledgments

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