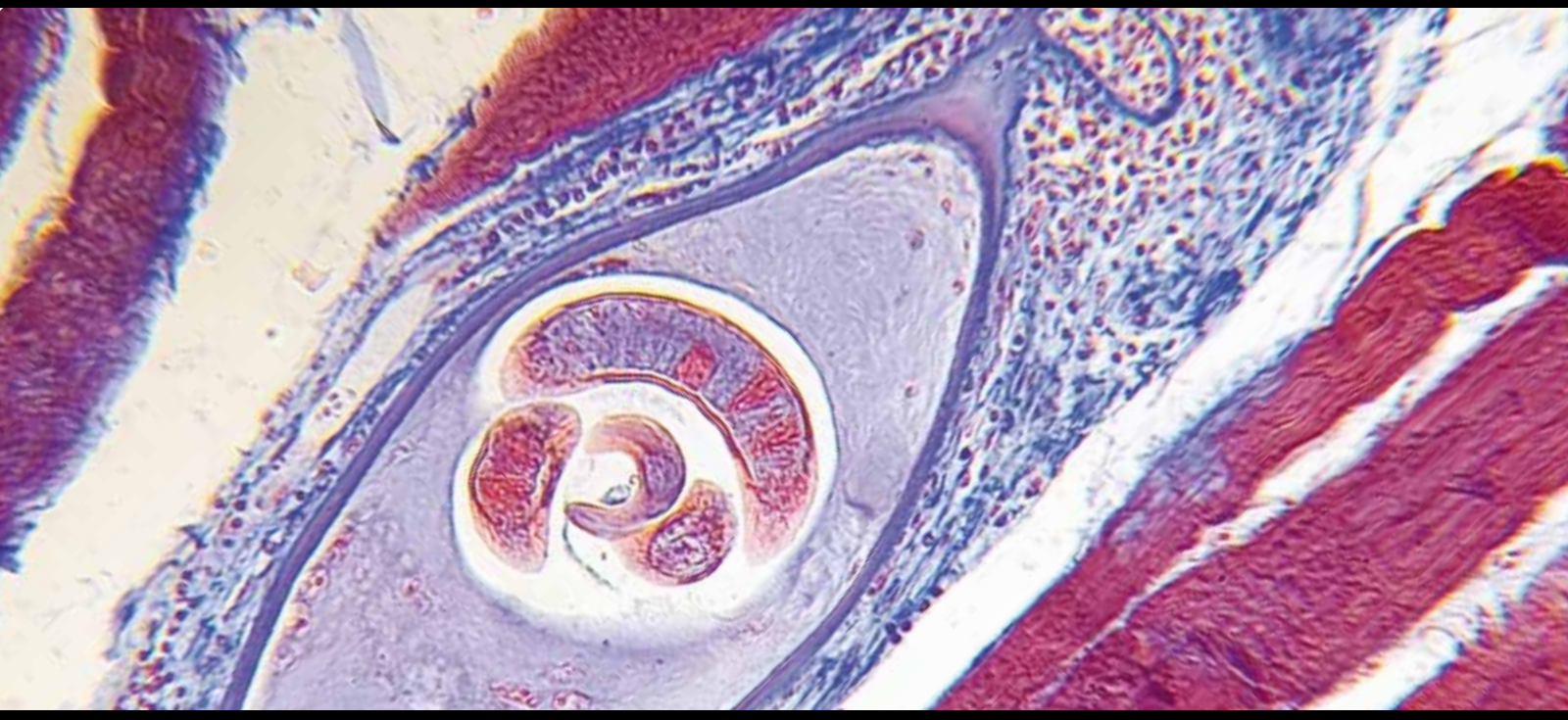
Gastroenterology Research and Practice

Novel Molecular Targets in Malignant Diseases of Digestive System

Guest Editors: Chunping Jiang, Youmin Wu, Qiang Xia, and Qin Huang



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Editorial Board

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Editorial

Novel Molecular Targets in Malignant Diseases of Digestive System

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Digestive malignancies remain one of the leading causes of cancer-related death worldwide despite the fact that increasing clinical and biological knowledge has emerged. Among all the newly discovered cancer cases, colorectal cancer, stomach cancer, liver cancer, and esophagus cancer rank in the front and the mortality rate for them also tops the list. The poor prognosis of digestive tumors is partially due to late diagnosis, delayed initiation of treatment, and unsatisfactory reaction to cancer therapies. Many molecular markers have been discovered for early diagnosis and better therapeutic outcomes from which we do benefit a lot. However, diagnosis and treatment of alimentary cancers require further optimization. We believe further study of novel molecular targets for early detection and better treatment would be helpful to reduce mortality rate and to improve the prognosis of malignant diseases of digestive system.

In this current issue, we focus on recent advances in the research of novel molecular targets which would help reveal the possible mechanism of tumorigenesis, progression, metastasis, and recurrence of digestive malignancies. The potential value of these molecular targets in cancer therapy is also highlighted. We present 10 articles on novel molecular targets in digestive system of which six articles discuss the molecular markers in hepatocellular carcinoma, three articles discuss the molecular makers in gastric carcinoma, and one makes a comprehensive review on one anticancer target in digestive system cancer therapy.

In the paper entitled "H-Ras oncogene expression and angiogenesis in experimental liver cirrhosis," by G. Ö. Elpek et al. evaluated the relation between H-Ras expression and angiogenesis in liver cirrhosis which can progress to liver carcinoma. The oncogene H-Ras is elevated in liver cirrhosis and correlates significantly with angiogenesis.

"LEPREL1 expression in human hepatocellular carcinoma and its suppressor role on cell proliferation" by J. Wang et al. found that LEPREL1 was downregulated in hepatocellular carcinoma (HCC) tissues both in mRNA and protein levels, and the down-regulation was not associated with conventional clinical parameters of HCC. LEPREL1 could serve as a potential tumor suppressor gene by inhibiting HCC cell proliferation.

The research paper "Expression of potential cancer stem cell marker ABCG2 is associated with malignant behaviors of hepatocellular carcinoma" by G. Zhang et al. found that ABCG2 could probably function as a liver cancer stem cell marker because of its close relationship with tumorigenesis and also because it could promote cell proliferation, drug resistance, and metastasis. This molecule may represent an attractive target for the innovation of cancer stem cell-directed therapy for HCC.

In "Study of RNA interference targeting NET-1 combination with sorafenib for hepatocellular carcinoma therapy *in vitro* and *in vivo*," S. He et al. found that the interference of NET-1 could enhance the anticancer effect of sorafenib.

The interference of NET-1 could lead to impaired ability of proliferation and migration and could induce apoptosis in HCC cell line. NET-1 may be a promising molecular target to develop adjuvant therapy in combination with the only effective targeted drug, sorafenib for HCC.

Another two articles talked about the function of microRNA in HCC. X.-Y. Huang et al. showed that miR-29 was upregulated in HCC and correlated with poor outcomes of HCC. It might function by promoting cell proliferation and inhibiting cell apoptosis. The work by Z. Wang et al. clarified the association of miR-499 and miR-34b/c polymorphisms with susceptibility to HCC and got to the final conclusion that rs3746444 was not associated with susceptibility to HCC while rs4938723 was associated with increased HCC risk.

"Mast cells positive to tryptase and c-kit receptor expressing cells correlates with angiogenesis in gastric cancer patients surgically treated" by M. Ammendola et al. studied the angiogenesis in gastric cancer and found that MCTP and c-kitR-EC correlated positively with microvascular density. Drugs against c-kitR and tryptase could be promising agents in antiangiogenic therapy.

"Significance of glutathione peroxidase 1 and caudal-related homeodomain transcription factor in human gastric adenocarcinoma," J. J. Han et al. demonstrated GPX1 and CDX2 might participate in the carcinogenesis, differentiation, and progression of gastric adenocarcinoma, and CDX2 might be an independent prognostic factor.

"Indirect comparison showed survival benefit from adjuvant chemoradiotherapy in completely resected gastric cancer with D2 lymphadenectomy" by Q. Yang et al. confirmed the role of adjuvant chemoradiotherapy in D2-resected gastric cancer patients with discussion of underlying molecular mechanism of this benefit.

The review article "PP2A-mediated anticancer therapy" by W. Chen et al. made a general review of the tumor suppressor PP2A by focusing on PP2A structure and the possible mechanism of its participation in anticancer therapy.

In summary, this special issue presents several intriguing achievements in the field of novel molecular targets in digestive malignancies which we hope could be utilized in the future for early diagnosis and treatment.

Acknowledgments

We wish to express our appreciation to all the authors for their excellent contribution and to the editors for their hard work on this issue.

Chunping Jiang
Youmin Wu
Qiang Xia
Qin Huang

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Research Article

Mast Cells Positive to Tryptase and c-Kit Receptor Expressing Cells Correlates with Angiogenesis in Gastric Cancer Patients Surgically Treated

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Background. Angiogenesis is a complex process involved in both growth and progression of several human and animal tumours. Tryptase is a serin protease stored in mast cells granules, which plays a role in tumour angiogenesis. Mast cells (MCs) can release tryptase following c-Kit receptor (c-KitR) activation. *Method*. In a series of 25 gastric cancer patients with stage $T_3N_{2\text{-}3}M_0$ (by AJCC for Gastric Cancer 7th Edition), immunohistochemistry and image analysis methods were employed to evaluate in the tumour tissue the correlation between the number of mast cells positive to tryptase (MCPT), c-KitR expressing cells (c-KitR-EC), and microvascular density (MVD). *Results*. Data demonstrated a positive correlation between MCPT, c-KitR-EC, and MVD to each other. In tumour tissue the mean number of MCPT was 15, the mean number of c-KitR-EC was 20, and the mean number of MVD was 20. The Pearson test correlating MCPT and MVD, c-KitR-EC and MVD was significantly ($r = 0.64$, $P = 0.001$; $r = 0.66$, $P = 0.041$, resp.). *Conclusion*. In this pilot study, we suggest that MCPT and c-KitR-EC play a role in gastric cancer angiogenesis, so we think that several c-KitR or tryptase inhibitors such as gabexate mesilate and nafamostat mesilate might be evaluated in clinical trials as a new antiangiogenetic approach.

1. Introduction

Angiogenesis is a complex process involved in growth, invasion, and metastasis of several animal and human tumours [1–3]. Mast cells (MCs) intervene in this process releasing classical proangiogenic factors, such as vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), fibroblast growth factor-2 (FGF-2), and nonclassical proangiogenic factors, such as tryptase and chymase, stored in their secretory granules [4–8]. The role of MCs has been broadly studied in benign lesions, in animal's and human's cancers, such as keloids, mast cells tumours, head and neck,

colorectal, lung, and cutaneous malignancies, indicating that MCs density is highly correlated with the extent of tumour angiogenesis [9–13]. Recent data have shown that MCs density is correlated with angiogenesis and progression of patients with gastric carcinoma [14, 15]. However, no data have been published regarding the correlation between MCs positive to tryptase (MCPT), c-Kit receptor expressing cells (c-KitR-EC), and microvascular density (MVD) in gastric carcinoma tissue. In the present study, we have evaluated correlations between the number of MCPT, c-KitR-EC, and MVD in a series of 25 gastric carcinomas with stage $T_3N_{2\text{-}3}M_0$ (by AJCC for Gastric Cancer 7th Edition), by means of immunohistochemistry and image analysis methods.

2. Methods

2.1. Patients. The clinicopathological features of studied patients are summarized in Table 1. A total of 25 gastric cancer patients diagnosed with preoperative gastric endoscopy underwent curative resection. Surgical approach used was open total gastrectomy with D2 lymph node dissection [16]. Patients were staged according to the American Joint Committee on Cancer 7th edition (AJCC-TNM) classification [17]. We have selected patients with stages III A and III B to the aim to correlate if MCPT, c-KitR-EC, and MVD in primary tumour tissue were associated with nodal involvement. On the other hand, metastatic patients with stage IV have no indication of surgery [13]. All patients had no distant metastases on computed tomography (TC) of the thorax, abdomen, and pelvis. All samples evaluated in this study were of adenocarcinomas histological type. Full ethical approval and consent from individual patients were obtained to conduct the study.

Table 1: Clinicopathological features of patients.

	N
Overall series	25
Age	
<65	8
>65	17
Sex	
Male	15
Female	10
Tumour site	
Cardia	5
Lesser curvature	3
Greater curvature	4
Body and fundus	7
Pyloric area	6
TNM by AJCC stage and type by Lauren classification	
$T_3N_2M_0$	14
$T_3N_3M_0$	11
Intestinal type	16
Diffuse type	9
Histologic type	
Adenocarcinomas	25
Histologic grade	
G1	3
G2	8
G3	14

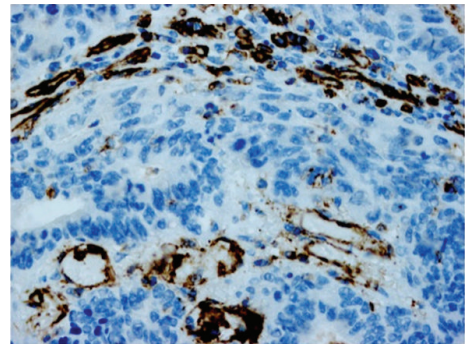
2.2. Immunohistochemistry. For the evaluation of MCPT, c-KitR-EC, and MVD, a three-layer biotin-avidin-peroxidase system was utilized [18]. Briefly, 4 μm thick serial sections of formalin-fixed and paraffin-embedded tumour samples were deparaffinised. Then, for antigen retrieval, sections were microwaved at 500 W for 10 min, after which endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Next, adjacent slides were subsequently incubated with monoclonal antibodies anti-CD31 (clone JC70a; Dako) diluted 1 : 40 for 30 min and pH 8 at room temperature, anti-c-KitR (CD117; Dako) for 30 min and pH 8, and anti-tryptase (clone 10D11; Novo Castra) diluted 1 : 150 for 20 min and pH 6 at room temperature. The bound antibody was visualized using biotinylated secondary antibody, avidin-biotin peroxidase complex, and 3-amino-9-ethylcarbazole. Nuclear counterstaining was performed with Gill's haematoxylin no. 2 (Polysciences, Warrington, PA, USA).

2.3. Morphometrical Assay. An image analysis system (Semiquantimet 400 Nikon) has been used. MCPT, c-KitR-EC, and MVD were observed at low magnification, and "hot spots" were selected at ×200 magnification [1, 17]. Areas of necrosis were not considered for counting. Hot spots were evaluated in three serial sections, and each single MCPT, c-KitR-EC, MVD was counted at ×400 magnification and reported as media from sections in order to avoid possible variability between sections.

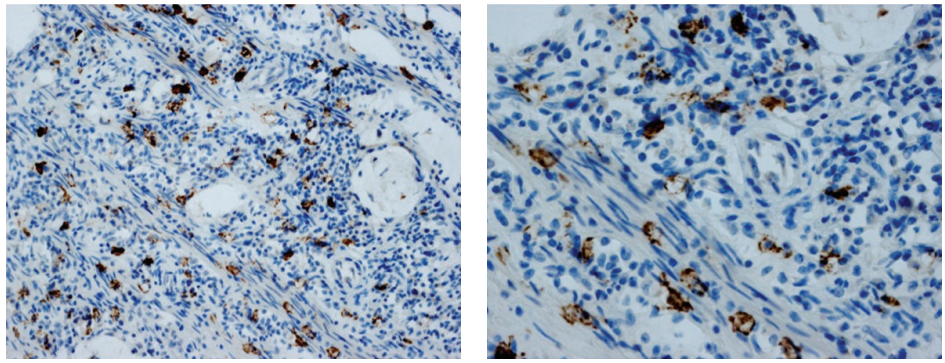
2.4. Statistical Analysis. Linear correlations between groups were quantified by means of the Pearson's correlation coefficient (r). t-test was used to statistically compare means. Correlation among MCPT, c-KitR-EC, MVD, lymph nodal involvement and the main clinical pathologial features were analysed by chi-square test. $P < 0.05$ was considered significant. All statistical analyses were performed with the SPSS statistical software package (SPSS, Inc., Chicago, IL).

3. Results

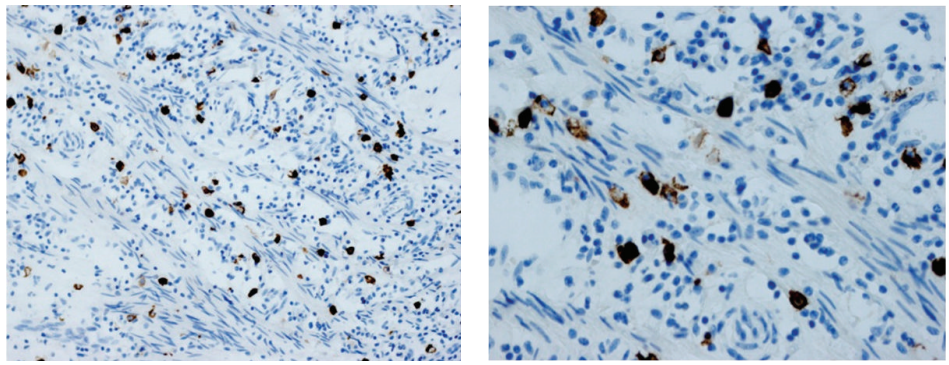
Immunohistochemical staining by antibodies anti-tryptase, anti-c-KitR and anti-CD31 allows the demonstration that MCPT and c-KitR-EC are well recognizable in highly vascularized gastric carcinoma tissue (Figure 1). Due to the possible interobserver variability at light microscopy in the evaluation of MCPT, c-KitR-EC, and MVD, the counts were performed by mean the above image analysis system. It is important to underline that the hot spots were evaluated at ×400 magnification in a well-reproducible microscopic area of 0.019 mm^2. In this manner, our results are related to



(a) Microvascular density (MVD) in gastric cancer sample stained with an anti-CD31 antibody (magnification: ×400)



(b) c-Kit receptor expressing cells (c-KitR-EC) in gastric cancer sample stained with an anti-c-KitR antibody (magnification: ×200; ×400)



(c) Mast cells positive to tryptase (MCPT) in gastric cancer sample stained with anti-tryptase antibody (magnification: ×200; ×400)

Figure 1: Representative immunohistochemical images relative to MCPT, c-KitR-EC, and MVD in gastric cancer tissue. Scale bar represents 100 μm.

an identified microscopic area. Considering all 25 samples (Table 1), in tumour tissue, the mean number of MCPT was 15, the mean number of c-KitR-EC was 20, and the mean number of MVD was 20. The Pearson test correlating MCPT and MVD and c-KitR-EC and MVD was significantly ($r = 0.64$, $P = 0.001$; $r = 0.66$, $P = 0.041$, resp.) (Figures 2 and 3, resp.). In the present study, clinicopathological features of patients were analysed, but no correlation among MCPT, c-KitR-EC, MVD, lymph nodal involvement and the main clinicopathological features was found.

4. Discussion

Up to now, the role of MCs in gastric cancer angiogenesis has not been clarified completely. We have a lot of data about the angiogenic process and its drug targets in tumours [19, 20], but there are few data on the role of MCs in gastric cancer angiogenesis [14, 21]. In particular, in a study designed by Mukherjee et al. [22], the authors studied MCs density in patients with gastric ulcers, well-differentiated cancers, and poorly differentiated cancers. The study was performed on

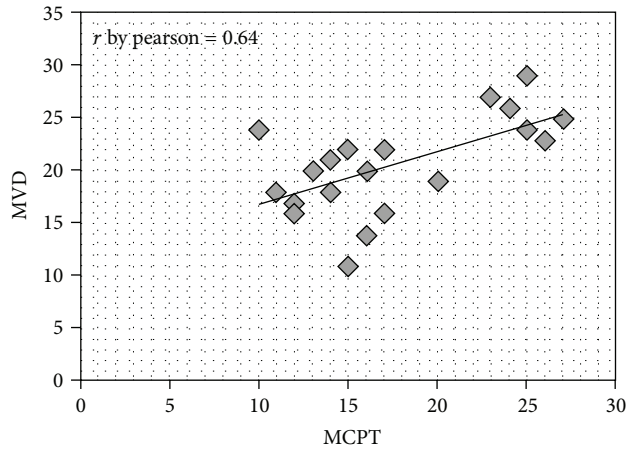


Figure 2: Pearson distribution of correlation between MCPT and MVD $r = 0.64$.

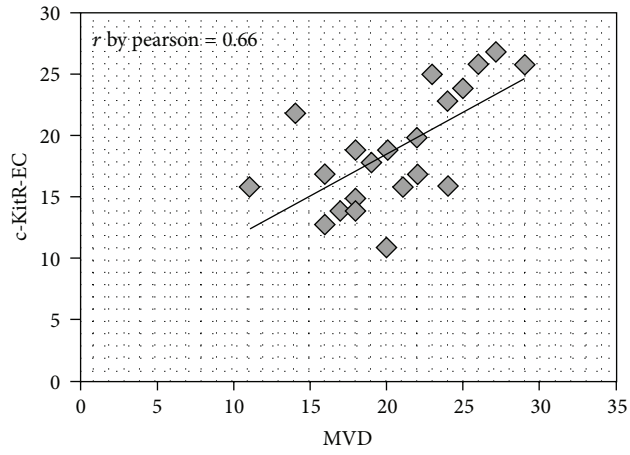


Figure 3: Pearson distribution of correlation between c-KitR-EC and MVD $r = 0.66$.

biopsies from gastric ulcers, well-differentiated cancers, and poorly differentiated cancers by means of toluidine blue stain. In this study, MCs density in well-differentiated was much higher than poorly differentiated carcinoma and correlated with angiogenesis.

Ribatti et al. [14] studied tumour samples from gastric cancer patients by means of immunohistochemistry employing anti-tryptase and anti-chymase antibodies to stain MCs found. In this study, a correlation between microvascular density and tryptase and chymase-positive masts cells with histopathological type was found.

Differences between the above studies and our results may be explained on the basis of different methods to identify MCs (toluidine blue, anti-tryptase antibody, and anti-chymase antibody), methods to assess MCs count (hot spots, random fields, and magnification), type of studied tissue (biopsy or surgically resected tumour), and stage of disease.

Taken together, these studies suggest that MCs are involved in gastric cancer angiogenesis. It is well demonstrated that tryptase is one of the most powerful angiogenic mediators released by human MCs following c-KitR activation and it may be angiogenic via several mechanisms. Tryptase is involved in tissue remodelling, and it may also act indirectly on tissue neovascularization by releasing latent angiogenic factors bound to the extracellular matrix [23–26]. Tryptase is an agonist of the proteinase-activated receptor-2 (PAR-2) in vascular endothelial cells. Activation of PAR-2 induces cell proliferation and the release of IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF), which, in turn, acts as angiogenic molecule [27].

In this pilot study, we found in a series of 25 surgical gastric cancer patients that MCPT and c-KitR-EC in tumour tissue, regardless of tumour staging or site, are positively correlated to the MVD. Our preliminary data suggest that MCPT and c-KitR-EC might play a role in gastric cancer angiogenesis. Further study in a large series of patients will be necessary to confirm first results. It is of interest underline that in this context, several c-KitR inhibitors such as imatinib mesilate or tryptase inhibitors such as gabexate mesilate and nafamostat mesilate [28, 29] might be evaluated in clinical trials as new antiangiogenetic strategy.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Research Article

LEPREL1 Expression in Human Hepatocellular Carcinoma and Its Suppressor Role on Cell Proliferation

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Background. Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies worldwide. It is characterized by its high invasive and metastatic potential. Leprecan-like 1 (LEPREL1) has been demonstrated to be downregulated in the HCC tissues in previous proteomics studies. The present study is aimed at a new understanding of LEPREL1 function in HCC. *Methods*. Quantitative RT-PCR, immunohistochemical analysis, and western blot analysis were used to evaluate the expression of LEPREL1 between the paired HCC tumor and nontumorous tissues. The biology function of LEPREL1 was investigated by Cell Counting Kit-8 (CCK8) assay and colony formation assay in HepG2 and Bel-7402 cells. *Results*. The levels of LEPREL1 mRNA and protein were significantly lower in the HCC tissues as compared to those of the nontumorous tissues. Reduced LEPREL1 expression was not associated with conventional clinical parameters of HCC. Overexpression of LEPREL1 in HepG2 and Bel-7402 cells inhibited cell proliferation ($P < 0.01$) and colony formation ($P < 0.05$). LEPREL1 suppressed tumor cell proliferation through regulation of the cell cycle by downregulation of cyclins. *Conclusions*. Clinical parameters analysis suggested that LEPREL1 was an independent factor in the development of HCC. The biology function experiments showed that LEPREL1 might serve as a potential tumor suppressor gene by inhibiting the HCC cell proliferation.

1. Introduction

HCC is one of the most prevalent tumors worldwide and the third leading cause of cancer-related deaths around the world [1, 2]. HCC is characterized by its high invasive and metastatic potential, rapid development, and poor prognosis. Currently, surgical resection, liver transplantation, and radiofrequency ablation have become the three validated curative treatments. However, even after the emergence of those auxiliary approaches, such as RFA or TACE, the 5-year tumor-free survival rate was reported to be approximately 50% with a 5-year tumor recurrence rate of more than 50% after resection [3, 4]. Liver transplantation is widely accepted to be the best method to approach the complete cure of HCC in selected patients. A study suggested that 5-year survival rates of patients fulfilling Milan criteria and patients fulfilling Hangzhou criteria is, respectively, 78.3% and 72.3% [5]. The presence of an unfavorable prognosis is mainly because HCC is a highly vascularized type of tumor with frequent intrahepatic or extrahepatic metastases. In recent years, remarkable progress has been made in the diagnosis and treatment of HCC, but the molecular mechanisms underlying HCC carcinogenesis remains unclear. Understanding the oncogenic role of genetic alteration that might happen during HCC progression is essential for development of innovative therapies.

Hepatocarcinogenesis is a complex multistep process in which many signaling cascades are altered. The most common mutations include the tumor suppressor gene TP53 (present in about 25–40% of the cancers, depending on the tumor stage) and CTNNB1 gene for β catenin (about 25%) [4, 6]. Epigenetic silencing of tumor suppressor genes, including

aberrant hypermethylation of CpG islands in promoters and histone modification, is considered a crucial event in the development and progression of tumors [7]. It has been reported that a number of tumor suppressor genes (ASC, CDH1, and RASSF1) are frequently inactivated in HCC [6, 8, 9]. Inactivation of these genes is usually detected with promoter CpG methylation and contribute to the process of carcinogenesis through cell proliferation promotion and apoptosis inhibition [10].

Leprecan-like 1 (LEPREL1) is a protein with an extensive similarity to the Gros1/Leprecan. This protein mainly concentrates in the endoplasmic apparatus and Golgi complex in the cell and is abundant in the basement membranes [11, 12]. Our previous study demonstrated that LEPREL1 was downregulated in the HCC tissues as compared to the adjacent nontumor tissues (data not shown). And LEPREL1 has been reported to suppress the proliferation of the breast cancer cell lines, which potentially makes them rather critical for the cancer diagnosis and treatment [13]. However, previous studies on LEPREL1 were seldom involved HCC. Therefore, this study was arranged to elucidate the correlation between LEPREL1 and HCC with the use of HCC specimens and cancer cell lines.

2. Materials and Methods

2.1. Patients and Clinical Samples. A total of 80 HCC patients who were treated with hepatectomy in our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China) in the second half of 2011 were enrolled in this study. The HCC was preoperatively diagnosed by appropriate imaging characteristics and was verified by histological examination after the operation. The cancer samples and matched noncancerous samples were obtained during the surgery. None of the patients received presurgical chemo- or radiation therapy. Clinicopathologic data were available for each of the 80 patients. Approval for these studies was obtained from the Institutional Review Board of the Faculty of our hospital in accordance with the ethical standards of the responsible committee on human experimentation as well as the declaration of Helsinki.

2.2. Cell Culture. Human HCC cell lines (HepG2 and Bel-7402) were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco-BRL), supplemented with 10% fetal bovine serum, in a 37°C incubator with 5% CO_2.

2.3. Total RNA Isolation, Reverse Transcription, and Real-Time PCR. Total RNA was isolated using TRIzol reagent (invitrogen) according to the protocols recommended by the manufacturer. The total RNA concentration and quantity were assessed by absorbance at 260 nm using Nano Drop ND-2000 spectrophotometer. A total of 1 μg of total RNA was used for the first-strand cDNA synthesis with PrimeScript reverse transcriptase (TaKaRa) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix PCR kit (TaKaRa) and an ABI PRISM 7500 Sequence Detector. Real-time PCR programs were as follows: one cycle of 95°C for 5 min, 95°C for 5 s, 50°C annealing for 30 s, and 72°C for 34 s, followed by 40 cycles. Three independent experiments were performed for each sample. The relative gene expression levels were determined using the 2-ΔΔCt method. The threshold cycle (CT) was measured during the exponential amplification phase and the amplification plots were analyzed by SDS 1.9.1 software (Applied Biosystems, Foster City, CA). Specific primer pairs were used for LEPREL1 (forward: 5′-ATGTGTGAGGGAACTTGCCACC-3′; reverse: 5′-TTGGCACACTCCAGGGCTTTCA-3′) and GAPDH (forward: 5′-CATCACCATCTTCCAGGAGCG-3′; reverse: 5′-TGACCTTGCCCACAGCCTT-3′).

2.4. Western Blot Analysis. 12 pairs of HCC samples (100 mg) or cells were lysed in the cold RIPA lysis buffer (Beyotime) supplemented with protease inhibitor phenylmethanesulfonyl fluoride (PMSF). The protein concentration was measured by bicinchoninic acid assay. Protein extracts (40 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels (Invitrogen). After electrophoresis, the separated proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Millipore) and were blocked with Tris-buffered saline (TBS) containing 5% nonfat milk for 1 h. The membranes were then incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal LEPREL1(Abcam), rabbit polyclonal GAPDH, rabbit polyclonal Cyclin A2, rabbit polyclonal Cyclin B1, rabbit polyclonal Cyclin D1, rabbit polyclonal Cyclin E2, rabbit polyclonal CDK2, and rabbit polyclonal CDK4 (Epitomics). Primary antibodies were diluted in TBST with 5% nonFAT milk at 1 : 1000. They were then incubated with a secondary goat anti-rabbit IgG monoclonal antibody (Epitomics) conjugated with horseradish peroxidase at 1 : 1000 dilution for 1 h at room temperature. Finally, the protein bands were detected by chemiluminescence using EZ-ECL chemiluminescent detection kit (BIOIND).

2.5. Immunohistological Chemistry Staining. The tissue samples of 86 patients with HCC were collected and fixed in 10% formalin before being embedded in paraffin. The tissue sections were cut at 4 μm and were dewaxed in 60°C incubator, followed by an absolute xylene rinse for 10 minutes. The sections were then rehydrated by serially rinsing the slides in 100%, 95%, 85%, and 75% ethanol for 5 min for each concentration. Antigen retrieval was performed by boiling the slides in the antigen retrieval buffer for 20 min, followed by natural cooling. The sections were blocked with 5% FBS-PBS solution for 30 min at 37°C and were incubated at 4°C overnight with rabbit polyclonal LEPREL1 antibody at 1 : 50 dilution. On the next day, the slides were incubated with the secondary antibody for 30 min at 37°C and the DAB was used for staining.

In order to evaluate the immunoreactivity of the LEPREL1 protein, a semiquantitative scoring method was used. The expression level of the LEPREL1-stained cells per field (×200) under microscope was calculated and was compared in different specimens by two separate observers in a double-blind

fashion. It was described as a score of 1 (<10% positive cells), 2 (10–20% positive cells), and 3 (>20% positive cells) [14].

2.6. Colony Formation Assay. After transfection with pcDNA3.1-LEPREL1 or control vectors for 24 h, the HepG2 and Bel-7402 cells were collected and placed onto the six-well plate (1000 cells per well). After a 14-day growth, the surviving colonies were fixed in methanol, washed two times with phosphate-buffered saline, dried, stained with liquid crystal violet (Sigma-Aldrich, Dorset), and counted. The experiments were repeated in triplicate.

2.7. Cell Counting Kit-8 (CCK8) Assay. HepG2 and Bel-7402 cells were plated in 96-well plates at the density of 5,000 cells per well with 100 μL of complete culture medium. After adhesion for 24 hours, the cells were transfected with pcDNA3.1-LEPREL1 or control vectors and were cultured for another 24, 48, 72, and 96 h. The wells where only the culture medium was added in them served as blanks. At each time point, the supernatant was removed and 100 μL of DMEM medium containing 10 μL of CCK8 (Dojindo) was added to each well for another 2 h at 37°C. The absorbance was recorded at 450 nm. All the experiments were independently repeated at least seven times.

2.8. Statistical Analysis. The SPSS 10.0 statistical analysis software was used to statistically process the experimental data. The CT values of LEPREL1 in tumor and nontumor samples and the effects of LEPREL1 on cell viability were compared using two-tailed Student's t-test. The clinicopathological features of the patients were compared using chi-square test (Fisher's exact test) for categorical variables. A P value of less than 0.05 indicated that the differences were statistically significant.

3. Result

3.1. The Expression of LEPREL1 in the HCC Tissues. In this study, we first detected the expression level of LEPREL1 using qRT-PCR in 80 pairs of HCC and the matched nontumor tissues. It was noticed that the downregulation of LEPREL1 was detected in 61/80 (76.3%) of the HCC tissues. The CT values of the LEPREL1 in tumor and nontumor tissues were then subjected to the appropriate statistical analysis. The results showed that the expression level of LEPREL1 was obviously lower in the tumor tissues as compared to that of the adjacent nontumor tissues ($P < 0.001$, $n = 80$, Figure 1(a)). The downexpression of LEPREL1 was also observed in the tumor tissues by western blot assay (Figure 1(b)).

The immunohistochemical staining was carried out on 86 HCC specimens and their corresponding adjacent noncancerous livers. As expected, the LEPREL1 expression was significantly lower in 68 (79.07%) of the 86 HCC specimens as compared with that of the adjacent noncancerous livers (Figure 1(c)). The examples of the positive immunostaining for LEPREL1 are shown in Figure 1(d).

3.2. The Correlation between LEPREL1 and Tumor Characteristics. The expression of LEPREL1 was analyzed in HCC with respect to several standard clinic-pathological features (Table 1). However, no significant difference was found between LEPREL1 expression and conventional clinic-pathological features, such as patient age, gender, HBV infection, vascular invasion, histological grade, or AFP level ($P > 0.05$).

Table 1: Clinicopathological characteristic of HCC patients.

	n	LEPREL1 score		P value
		+++	++~+	
Age				
≦60	56	15	41	0.096
>60	30	3	27	
Gender				
Male	75	14	61	0.231
Female	11	4	7	
HBsAg				
+	64	15	49	0.544
−	22	3	19	
Size of tumor				
≦5	36	4	32	0.066
>5	50	14	36	
Edmondson grade				
I + II	38	9	29	0.603
III + IV	48	9	39	
Vascular invasion				
Without	51	11	40	1.000
With	35	7	28	
Tumor number				
Single	77	14	63	0.087
Multiple	9	4	5	
Liver cirrhosis				
Without	43	12	31	0.184
With	43	6	37	
Coating				
Without	67	15	52	0.751
With	19	3	16	
AFP				
≧400	38	7	31	0.790
<400	48	11	37	

LEPREL1: leprecan-like 1; HBsAg: hepatitis B surface antigen; AFP: alpha fetoprotein.
The significance of the difference between groups in the table was assessed by chi-squared tests (Fisher's exact test).

3.3. Effects of LEPREL1 on Cell Viability. To investigate the tumor suppression ability of LEPREL1 in the HCC cells, we observed the effect of LEPREL1 expression on cell proliferation and colony formation. Cell growth assay showed that the growth of HepG2 and Bel-7402 cells was significantly reduced after the transfection with pcDNA3.1-LEPREL1 as compared to that of the cells transfected with an empty pcDNA3.1 vector ($P < 0.05$, Figure 2(a)). As shown in Figure 3,

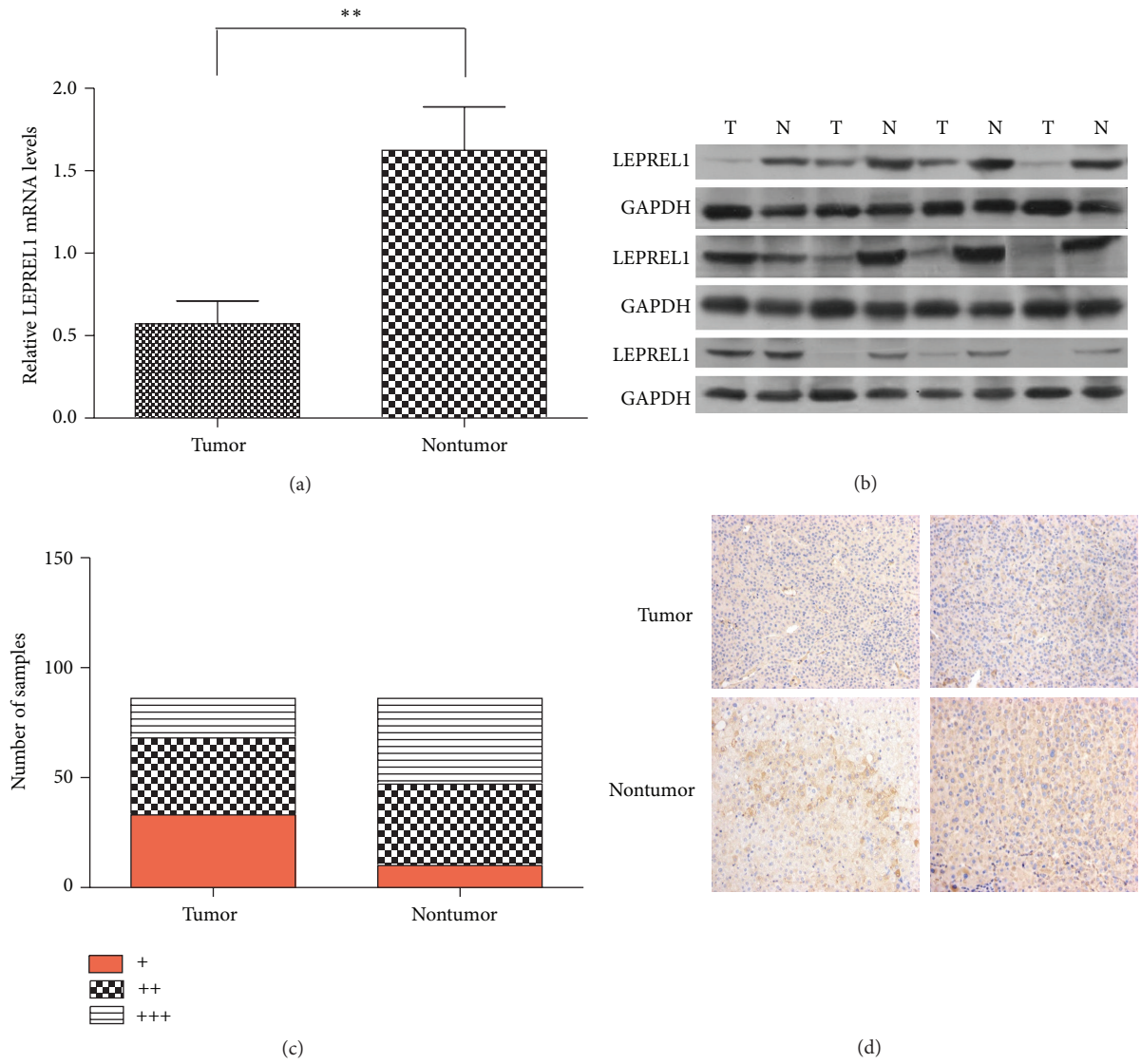

Figure 1: LEPREL1 was frequently downregulated in HCC. (a) The expression level of LEPREL1 relative to GAPDH was compared between the nontumorous and tumor tissues in 80 HCCs using quantitative RT-PCR. Expression of LEPREL1 in tumor tissues was significantly lower than that of the nontumorous tissues ($P < 0.001$, $n = 80$). (b) Representative pictures of LEPREL1 protein expression in randomly selected paired HCC nontumor and tumor tissues. GAPDH was used as an endogenous control. LEPREL1 was downregulated in most of the tumor tissues as compared to the adjacent nontumorous tissues. ((c), (d)) Expression of LEPREL1 in the tumor tissues and adjacent nontumor tissues. Representative pictures of the immunohistochemistry results of LEPREL1 in tissues (d) and histogram of semiquantitatively with a three-tiered system grades ($n = 86$). N: nontumor, T: tumor.

the frequency of the colony formation in the cells transfected with pcDNA3.1-LEPREL1 was significantly lower than that of the cells with an empty pcDNA3.1 vector. According to our observations in these assays, their ability to suppress the proliferation could imply that LEPREL1 might have the potential to act as a tumor suppressor (Figures 2(b) and 2(c)).

3.4. LEPREL1 Inhibit Cell Proliferation by Modulating Cell Cycle Regulatory Proteins in HepG2 Cells. To understand the potential mechanism of LEPREL1 on inhibiting HCC cell lines proliferation, the expression of major cell cycle regulatory proteins including Cyclins A2, B1, D1, E2, CDK2, and CDK4 was assessed by western blot. The cells transfected with pcDNA3.1-LEPREL1 exhibited a significant decrease in the levels of Cyclin A2 and Cyclin E2 expression when compared with the control but had no detectable effect on Cyclin D1, Cyclin B1, CDK2, and CDK4 expression (Figure 3).

4. Discussion

There have been no previous reports describing the LEPREL1 expression patterns in the HCC. In the present study, we found that LEPREL1 was frequently downregulated in HCC. The ectopic expression of LEPREL1 could suppress the proliferation and colony formation, implying that they might play an important role in the HCC progression.

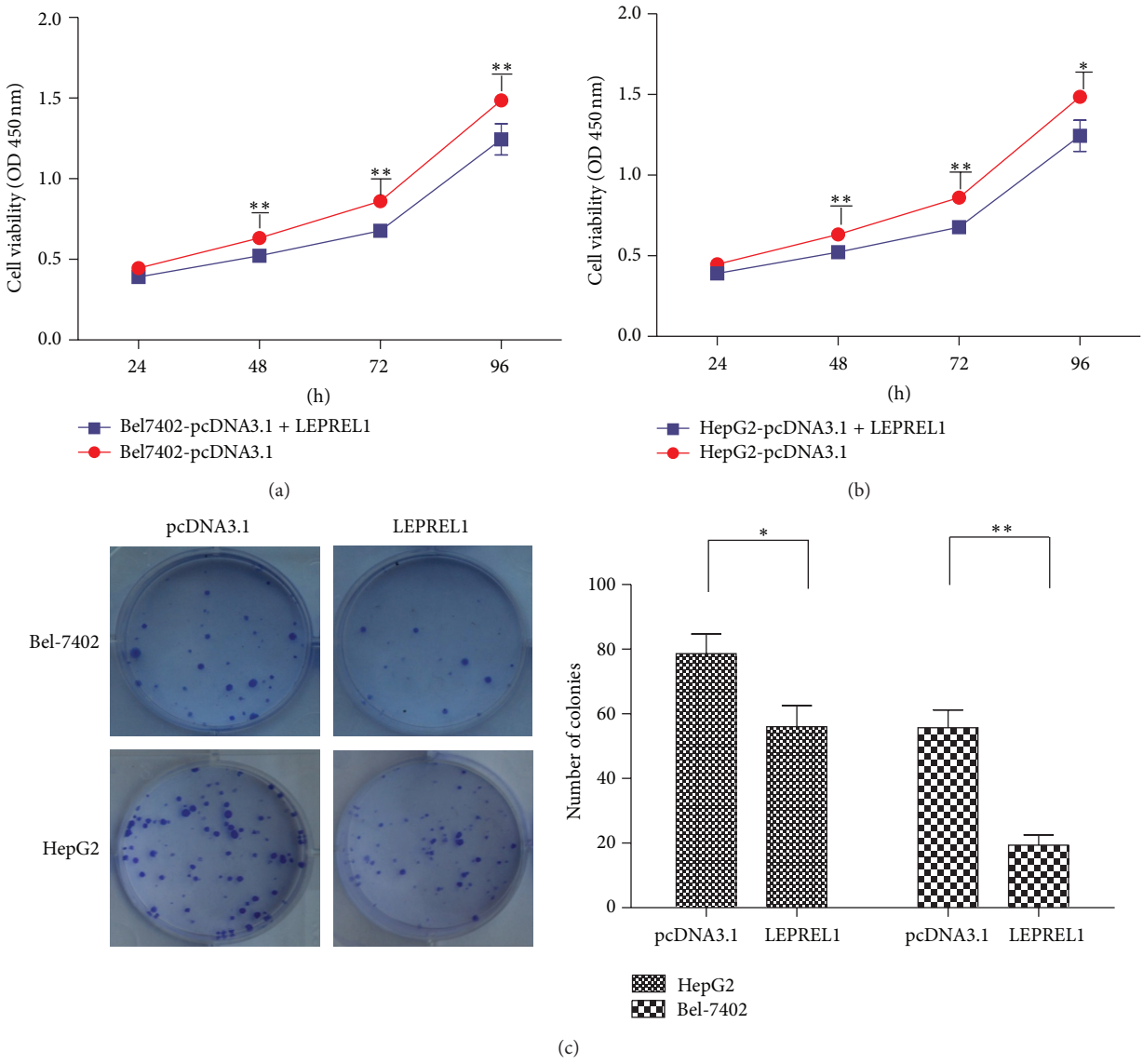

Figure 2: The effect of LEPREL1 on cell growth and colony formation. ((a) and (b)) Exogenous LEPREL1 was expressed in Bel-7402 (a), HepG2 (b) cells transfected with the pcDNA3.1 vector. Parental cells with an empty vector were used as a control. A t-test was used to show significant differences between the two groups ($P < 0.05$). (c) To observe the effects of LEPREL1 on colony formation, pcDNA3.1-LEPREL1 was transfected into Bel-7402 and HepG2 cells. Twenty-four hours after the transfection, the cells were plated on the dishes and were cultured in G418 for two weeks. Representative photographs of the colony formation from different stable cell lines are shown in the left panels. The colony formation rate (%) is shown in the right panels (calculated by dividing the colony numbers by 1×10^3 plated cells). The data (mean ± SEM) were obtained from three independent experiments ($^*P < 0.05$; $^{**}P < 0.01$). The representative dishes showed the inhibitory effects of LEPREL1 on colony formation. The histogram shows that colony formation was significantly suppressed by LEPREL1 as compared with the empty vector control, where the numbers are the mean value of three independent experiments with SD.

LEPREL1 is a member of prolyl 3-hydroxylases family, which belongs to the family of 2-oxoglutarate dioxygenases. The posttranslational modifications of the collagen including biosynthesis, folding, and assembly are dependent on 2-oxoglutarate dioxygenases. The collagen prolyl 3-hydroxylases catalyze the 3-hydroxylation of different kinds of collagens, which especially occurs in types IV and V collagens [15]. Furthermore, LEPREL1 has been detected in the tissues rich in basement membranes and was reported to participate in the hydroxylation of collagen IV [12]. Type IV collagen along with laminin, perlecan, and nidogen is major components of the basement membrane (BM). The basement membrane is a complex network of interacting proteins, including type IV collagen (Col IV) that acts as a scaffold to stabilize the physical structures of tissues. Type IV collagen also plays an important role in cell adhesion, migration, proliferation, differentiation, and tumor angiogenesis [16]. Moreover, the BM is a physical barrier that prevents tumor invasion. Impaired expression of type IV collagen has been reported to be an early event in the acquisition of an invasive

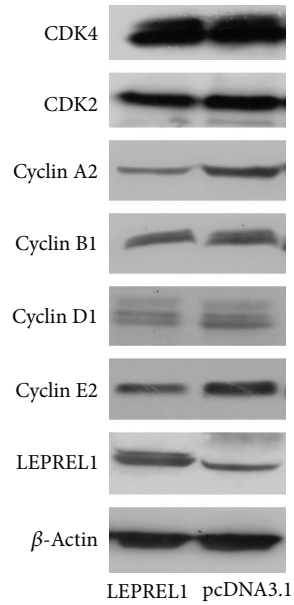

Figure 3: Cell cycle regulatory proteins expression analysis in HepG2 cells treated without (control) and with LEPREL1 for 72 h.

phenotype in some epithelial cancers [17]. Another study has demonstrated that the native Col IV induced an EMT-like process in the MCF10A human mammary nontumorigenic epithelial cells [18]. We speculated that a low expression of P3H2 could affect the properties of the basement membrane, which could facilitate the degradation by the enzyme secreted by tumor cells. There is a need for further investigation to elaborate on the functions of LEPREL1 in cancer invasion.

In addition to the potential functions of LEPREL1 on invasion and metastasis in carcinoma cells, our results demonstrated that LEPREL1 had a direct antiproliferative effect in HCC. Therefore, it was concluded that the implications of this gene might cause tumor suppression. The epigenetic silencing of the tumor suppressor genes could be considered to be a major event contributing to the development and progression of human cancers. Hypermethylation of the DNA cytosine residues at the carbon 5 position (5 mC) in the CpG islands in intragenic, promoter, and intergenic regions is a common epigenetic mechanism in the eukaryotic DNA, which plays an important role during differentiation and in response to some types of physiological changes. Increasing evidence has shown that DNA methylation could be involved in genomic instability and silencing of the tumor suppressor genes in many cancers, including HCC [19–21]. Recently, there has been a number of studies reporting an aberrant methylation of the genes such as GSTP1, RASSF1A, and APC, which has been detected in HCC [22, 23]. The loss of function of these genes as a result of the hypermethylation of the CpG islands in promoters might contribute to the progression of the tumors. It has been reported that the inactivation of LEPREL1 in breast cancer has been attributed to an aberrant CpG methylation in the $5'$ regulatory sequence of LEPREL1. Furthermore, the methylation of the LEPREL1 CpG island was specific to the oestrogen receptor-positive breast cancers [13]. The detailed mechanism of the LEPREL1 downregulation in HCC needs further investigation to elucidate whether it could be attributed to the methylation of the CpG islands in the promoter region.

The antiproliferation mechanism of LEPREL1 was further studied by detecting cell cycle regulatory proteins. Overexpression of these cyclins and CDKs altered the cell cycle progression which is closely associated with malignancy. Many studies suggested that overexpression of Cyclins D1, E, and CDK4 protein levels resulting in uncontrolled cell proliferation is closely associated with HCC [24, 25]. Cyclin D1 is a major mitogen-induced regulator of cell cycle progression that has a central function in regulating G1 progression and forms a complex with and functions as a regulatory subunit of CDK4 or CDK6 [26]. Cyclin E/CDK2 complexes have a pivotal role in G1 to S phase transition [27]. Cyclin A2 binds and activates CDC2 or CDK2 kinases and thus promotes both cell cycle G1/S and G2/M transitions. Cyclin B1 expresses predominantly during G2/M phase and forms a cell cycle-dependent complex with p34(cdc2) to promote mitosis [28]. Our results shown that epigenetic expression of LEPREL1 inhibitS the cancer cell proliferation by arresting the G1/S phase via downregulation of cell cycle regulatory proteins, including Cyclin A2 and Cyclin E2.

With a cohort of 86 randomly selected HCC patients, we investigated the potential downregulation of the LEPREL1 with numerous clinical parameters, including age, gender, tumor size, Edmondson grade, vascular invasion, and AFP. However, the expression of the LEPREL1 was not associated with any of the above clinical parameters.

5. Conclusions

This study is the first to examine the role of LEPREL1 in HCC and reveals that the LEPREL1 played a key role in proliferation inhibition of the HCC cell lines. Our results

suggested that the LEPREL1 might be a tumor suppressor gene.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

PP2A-Mediated Anticancer Therapy

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PP2A is a family of mammalian serine/threonine phosphatases that is involved in the control of many cellular functions including protein synthesis, cellular signaling, cell cycle determination, apoptosis, metabolism, and stress responses through the negative regulation of signaling pathways initiated by protein kinases. Rapid progress is being made in the understanding of PP2A complex and its functions. Emerging studies have correlated changes in PP2A with human diseases, especially cancer. PP2A is comprised of 3 subunits: a catalytic subunit, a scaffolding subunit, and a regulatory subunit. The alternations of the subunits have been shown to be in association with many human malignancies. Therapeutic agents targeting PP2A inhibitors or activating PP2A directly have shed light on the therapy of cancers. This review focuses on PP2A structure, cancer-associated mutations, and the targeting of PP2A-related molecules to restore or reactivate PP2A in anticancer therapy, especially in digestive system cancer therapy.

1. Introduction

Protein phosphatase 2A(PP2A) is a member of phosphoprotein phosphatase (PPP) family which belongs to the superfamily of protein serine/threonine phosphatases that reverse the actions of protein kinases by cleaving phosphate from serine and threonine residues of proteins. It has been proven that PP2A regulates various cellular processes, including protein synthesis, cellular signaling, cell cycle determination, apoptosis, metabolism, and stress responses [1–3]. PP2A is widely described as a tumor suppressor since the first recognition that its inhibitor okadaic acid is a tumor promoter, and mutations of PP2A subunits can be detected in a variety of human malignancies. The tumor suppressing function of PP2A makes it a possible target in anticancer therapy.

Colorectal cancer is the third most common cancer in males and the second in females, and about 25% of patients with colorectal cancer present with overt metastatic disease. Forty to 50% of newly diagnosed patients can develop metastasis [4, 5]. Liver cancer is the fifth most common cancer in males and the seventh most in females worldwide. It ranks the third in cancer-related deaths [5]. Hepatocellular carcinoma (HCC) which account for 70–85% of primary malignancies in liver is the dominant histological type of primary liver cancer [6]. To date, the treatment of these two cancers is not satisfactory, and the discovery of new therapeutic agents is in demand. Among all the possible targets, PP2A is a promising one.

In this review, we focus on the structure of PP2A and the possible mechanism of its participation in anticancer therapy with special emphasis on targeting PP2A in colorectal cancer and HCC.

2. PP2A Structure and Cancer-Associated Mutations

The holoenzyme structure of PP2A comprises a 36 kDa catalytic subunit (PP2AC or C subunit), a 65 kDa scaffolding subunit (PR65 or A subunit), and a regulatory subunit (B subunit). A C subunit and an A subunit make the PP2A core enzyme (PP2AD) which then binds with a B subunit, thus, making the PP2A heterotrimeric holoenzyme (PP2AT).

The catalytic subunit PP2AC is comprised of 309 amino acids and has two different isoforms (α and β) which are encoded by two separated genes but share 97% sequence

Table 1: Nomenclature of subunits of PP2A and the subcellular distribution.

Subunit	Gene name	Gene locus	Isoforms	Aliases	Subcellular distribution	References
A	PPP2R1A	19q13.14	Aα	PR65α	Cytoplasm	[1, 25, 54, 55]
	PPP2R1B	11q23.1	Aβ	PR65β	Cytoplasm	[1, 13, 25, 54]
B	PPP2R2A	8p21.1	Bα	PR55α, B55α	Cytoplasm, microtubules, neurofilaments, vimentin, membrane, nucleus, Golgi/reticulum	[1, 25, 54]
	PPP2R2B	5q32	Bβ	PR55β, B55β	Cytosol	[1, 25, 54, 56]
	PPP2R2C	4p16.1	Bγ	PR55γ, B55γ	Cytoskeletal fraction	[1, 25, 54, 57]
	PPP2R2D	10q26.3	Bδ	PR55δ, B55δ	Cytosol	[1, 25, 54]
B′	PPP2R5A	1q32.3	B′α	PR61α, B56α	Cytoplasm	[1, 25, 54]
	PPP2R5B	11q13.1	B′β	PR61β1, B56β, PR61β2	Cytoplasm	[1, 25, 54, 58]
	PPP2R5C	14q32.31	B′γ1B′γ2B′γ3	PR61γ1, B56γ1, B′α3 PR61γ2, B56γ2, B′α2 B56γ3, B′α1	Cytoplasm, nucleus, focal adhesion	[1, 25, 54]
	PPP2R5D	6p21.1	B′δ	PR61δ, B56δ	Cytosol, mitochondria, nucleus, microsomes	[1, 25, 54]
	PPP2R5E	14q23.2	B′ε	PR61ε, B56ε	Cytoplasm	[1, 25, 54]
B″	PPP2R3A	3q22.1	B″α1B″α2	PR130 PR72	Centrosome and Golgi Cytosol	[1, 25, 54, 59]
	PPP2R3B	Xp22.33	B″β1B″β2	PR48 PR59	Nucleus	[1, 25, 54]
	PPP2R3C		B″γ	G5PR	Nucleus	[1, 25, 54]
B‴	STRN	2p22.2		PR110, PR93	Membrane and cytoplasm	[1, 25, 54]
	STRN3	14q12		PR112, PR102,PR94	nucleus	[1, 25, 54]
C	PPP2CA	5q31.1	Cα	PP2Aα	Cytoplasm and nucleus	[1, 25, 54]
	PPP2CB	8p12	Cβ	PP2Aβ	Cytoplasm and nucleus	[1, 25, 54, 60]

similarity. Despite the sequence similarity, PP2ACα and PP2ACβ seem to not be able to compensate for each other because PP2ACα knockout mice cannot survive. PP2AC is highly expressed in hearts and brains and is mainly distributed in cytoplasm and nucleus. The regulation of PP2AC is highly organized and precise which is usually made up of phosphorylation at Tyr307 and Thr304 and methylation at Leu309. Phosphorylation at Thr304 is regulated by autophosphorylation-activated protein kinase and can inhibit the recruitment of B55 subunits [7, 8]. Thr307 can be phosphorylated by p60v-src as well as by other receptor and nonreceptor tyrosine kinases which results in a decrease of phosphatase activity and thus can inhibit the interaction with B56 subunits and B55 subunits [9]. The posttranslational modification with methylation at Leu309 is catalyzed by leucine carboxyl methyltransferase 1 (LCMT1) and PP2A methylesterase-1 (PME-1). The methylation can enhance the affinity of PP2A for B55 subunits which can be reversed by phosphorylation at Tyr307 [10] (Table 1).

The A subunit serves as a structural subunit and can bind to a C subunit with its C-terminal repeats 11–15 and to a B subunit with its N-terminal repeats 1–10. The A subunit structure is composed of 15 tandem repeats of a 39 to 41 amino-acid sequence which is called HEAT (huntingtin/elongation/A subunit/TOR) motif. The HEAT repeats of the scaffold A subunit form a horseshoe-shaped fold, holding the catalytic C and regulatory B′ subunits together on the same side [11]. Like the C subunit, the A subunit is also composed of two isoforms (α and β) which share an 87% sequence similarity, and both are widely expressed in cytoplasm. Despite the consistency in the sequence, the 2 isoforms are functionally distinct and cannot substitute for each other, because overexpressed Aα fails to revert the transformed phenotype in Aβ suppressed cells [12]. Unlike Aα, which is ubiquitously expressed in different tissues and cells, the Aβ expression level varies and can sometimes be detected with mutations in tumor tissues with a more common frequency. Mutations of both genes are found to occur at low frequency in human tumors. The gene encoding Aβ was founded to be altered in 15% of primary lung cancers, 15% of colorectal cancers, and 13% of breast cancers, making it unable to bind to B and/or C subunits *in vitro* [13–15]. The alternations include gene deletion, point mutation, missense, and frameshifts. Sablina et al. found that loss of Aβ can permit immortalized human cells to achieve a tumorigenic state and contribute to cancer progression through dysregulation of small guanosine triphosphatase (GTPase) RalA activity which can be dephosphorylated by Aβ at Ser183 and Ser184 and is thus a necessity for the transformed phenotype induced by suppression of Aβ [12, 16]. The Aα gene alternations can also be found in a variety of neoplasms, like melanomas, breast cancers, and lung cancers, though in a lower frequency when compared with Aβ [14, 17]. To date, 4 kinds of caner-associated mutation of Aα have been detected: E64D, E64G, R418W, and Δ171-589 [17]. The specific binding of SV40 small t (ST) antigen to Aα can lead to the elimination of its capacity to from complex with B56γ which

Table 2: Cancer-associated mutations of PP2A A subunits.

Subunit	Mutation name	Alternations	Consequence	Cancer type	References
PR65α	E64D E64G R418W	Point mutation	Deficiency in binding to B$'\alpha$1 Deficiency in binding to B/C subunits	Breast Lung Skin	[15, 17, 61]
	Δ171-589	Deletion		Breast	
PR65β	G8R P65S G90D L101P L101P/V448A K343E V448A D504G V545A	Missense	Dysregulation of RalA GTPase, leading to impaired binding capacity to B/C subunits	Lung Breast Colon	[12, 15, 43]
	ΔE344-E388	In-frame deletion			[12, 15, 43]

results in human cell transformation [18]. By introducing Aα mutants into immortalized but nontumorigenic human cells, Chen et al. found that Aα mutants can induce functional haploinsufficiency which can somehow lead to the deficiency to dephosphorylate Akt. Then, the active form of Akt results in the human cell transformation [19] (Tables 1 and 2).

The regulatory B subunits are encoded by 4 unrelated gene families: PR55/B (PPP2R2A, PPP2R2B, PPP2R2C, PPP2R2D), PR56/61/B$'$ (PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, PPP2R5E), PR130/72/48/59/G5PR/B$''$ (PPP2R3A, PPP2R3B, PPP2R3C), and PR93/110/B$'''$ (STRN, STRN3), and each member from the 4 families shows no similarity in sequence. The B family has 4 isoforms: α, β, γ, and δ. They all show a time and space expression pattern: the α and δ isoforms are widely distributed in tissues, while the β and γ isoforms are enriched in the brain. The expression level of the β isoform decreases while γ elevates. The mainly subcellular distribution of these 4 isoforms are cytoplasm/nucleus, cytosol, cytoskeletal fraction, and cytosol, respectively [1]. The B$'$ family has 5 isoforms: α, β, γ, δ, and ε. Like the B family, they are also expressed and enriched in certain tissues and subcellular cavity. All B$'$ family members contain a highly conserved central region which is 80% identical and is responsible for the interaction with A/C subunits and a divergent C-terminal and N-terminal which may confer different functions, such as regulation of substrate specificity and subcellular targeting [20]. The B$''$ family contains 5 isoforms that might arise from the same gene by alternative splicing. PR 130 is widely expressed in all tissues and enriched in the heart and muscle, while PR72 is exclusively expressed in the heart and muscle. PR48 shares 68% homology with PR59 and is mainly distributed in nucleus. It is an interaction partner of Cdc6 which functions in the initiation of DNA replication. PR59 is believed to be an interaction partner of p107 protein, and when overexpressed, it can bind to and dephosphorylate p107 protein, thus, leading to the expression of DNA damage related genes which induces inhibition of cell cycle progression [21]. The B$'''$ family contains newly identified members that share a conserved epitope with the B$'$ family. PR93, also termed S/G2 nuclear autoantigen (SG2NA), is mainly distributed in the brain and muscle while PR110, also termed striatin mainly in the postsynaptic densities of neuronal dendrites [22]. They both can act as a calmodulin binding protein to interact with PP2AC in a calcium-dependent manner. It is believed that the variety of B subunits accounts for the functional specificity of PP2A. Besides the cancer-associated mutations of the A subunits, the alternations of B subunits can also contribute to cell transformation. Alternation of certain types of B subunits have been detected in neoplasms, like the decreased expression level of B56γ in human melanoma cell lines [23]. Ito et al. reported that a truncated B56γ1 isoform which can disrupt PP2A phosphatase activity *in vivo* is expressed in a metastatic clone, BL6, of mouse B16 melanoma cells and is sufficient to enhance the metastasis of another clone, F10 [24]. The overexpression of PR65γ in human embryonic kidney epithelial cells and human hepatocellular cell lines can revert the cell transformation [18] (Table 1).

3. Reactivate PP2A to Augment Anticancer Effect by Targeting Inhibitory Proteins of PP2A

To date, 4 kinds of cellular inhibitory proteins of PP2A has been described, namely, CIP2A, pp32/I_1^{PP2A}, SET/I_2^{PP2A}, and SETBP1. Compared with environmental toxins like okadaic acid, they are more selective. Emerging studies suggest that aberrant expression and/or activity of these phosphatase inhibitors may be associated with many human malignancies (Table 3).

3.1. CIP2A. The increased expression of cancerous inhibitor of PP2A(CIP2A) has been described in many kinds of malignancy like HCC, breast cancer, colorectal cancer, ovarian cancer, cervical cancer, prostate cancer, lung cancer, chronic myeloid leukemia, and acute myeloid leukemia [25]. In nonsmall cell lung cancer, CIP2A elevation correlated with

Table 3: Inhibitory protein of PP2A and possible related anticancer drugs.

Inhibitory protein	Interaction with PP2A	Drugs against inhibitory	References
CIP2A	Prevent PP2A-dependent dephosphorylating of c-Myc	Bortezomib; Erlotinib	[32–35]
PHAPI/pp32/I_2^{PP1A}	Direct binding	Jacalin	[39, 40]
SET/I_2^{PP2A}	Direct binding	COG112; Apolipoprotein E-mimetis peptides	[39, 43, 44]
SETBP1	Form a SETBP1-SET-PP2A complex		[45]

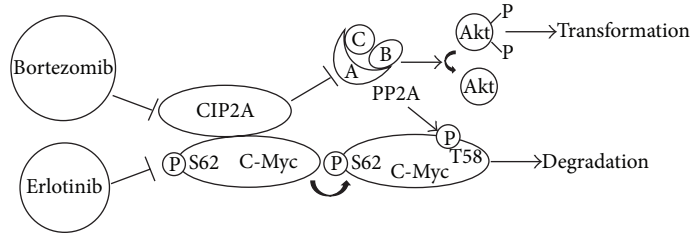

Figure 1: Bortezomib and erlotinib restore PP2A activity by targeting CIP2A. Bortezomib and erlotinib downregulate CIP2A which leads to up-regulation of PP2A activity and therefore inhibiting cell transformation by inactivating Akt.

elevated C-Myc expression levels, and is a significant prognostic predicator for poor survival [26]. Likewise, in acute myeloid leukemia, prostate cancer, and other malignancies, increased CIP2A predicts poor differentiation and worse consequences [27, 28].

Many studies have demonstrated that the disruption and dysfunction of PP2A is a requirement for malignant transformation. Because of PP2A's multiple functions in pathway regulations and variant functions attributed to different PP2A subunits, the mechanism of induced cell transformation is distinct and complicated, such as the dysregulation of Wnt/β-catenin signaling pathway and the Bcl-2 family of apoptosis regulators as well as the deficiency in inhibiting the oncogenic transcription factor c-Myc [29]. C-Myc has two phosphorylation residues: Ser62 and Thr58. The phosphorylation of Thr58 permits the dephosphorylation by PP2A on Ser62 which lead to further degradation of C-Myc. In human cell transformation, inhibition of PP2A fails to dephosphorylate c-Myc Ser62, making it overexpressed in malignancies [30]. The possible mechanism underlying the inhibition of PP2A-mediated c-Myc Ser62 dephosphorylation has been interpreted by Junttila et al. The PP2A inhibitor CIP2A can act as the c-Myc stabilizing protein. It can directly bind to c-Myc through recognition of the Ser62 site and then prevent PP2A-dependent dephosphorylation of c-Myc [31].

In consideration of its inhibition of PP2A in the stabilization of c-Myc and other signals like the Akt signal, CIP2A has be found to be an anticancer target. CIP2A is reported to be a target of bortezomib in many kinds of malignancies, such as HCC, leukemia, human triple negative breast cancer, and head and neck squamous cell carcinoma [32, 33]. Chen et al. found that bortezomib can downregulate CIP2A in a dose- and time-dependent manner, and can upregulate PP2A activity in HCC. The inhibition of CIP2A by bortezomib leads to PP2A-dependent Akt inactivation and tumor cell apoptosis [34]. The apoptotic effect of bortezomib is also described in leukemia cells by downregulation of CIP2A and upregulation of PP2A activity [35]. Bortezomib can sensitize solid tumor cells to radiation through the inhibition of CIP2A [36]. Besides apoptosis-inducing role of bortezomib by antagonizing CIP2A, the induced autophagy by bortezomib also depends on the down-regulation of CIP2A and p-Akt in HCC [37]. And in sensitive hepatocellular cells, the apoptosis-inducing effect of erlotinib is mediated by down-regulation of CIP2A besides its status as a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). The newly discovered effect of erlotinib by CIP2A-dependent p-Akt down-regulation makes CIP2A a possible target in the treatment of HCC [38] (Figure 1).

3.2. PHAPI/pp32/I_1^{PP2A}. The protein putative human HLA-DR-associated protein I(PHAPI), which has been variously identified as pp32 or I_1^{PP2A}, is a putative HLA class II-associated cytosolic protein and also a potent tumor suppressor. It has been shown to be a PP2A inhibitor though the detailed mechanism has not been fully understood, probably by binding directly to the C subunit [39]. Yu et al. found that the antiproliferative lectin, jacalin, can dissociate PP2A from PHAPI through inducing tyrosine phosphorylation of PHAPI in HT29 colon cancer cells [40]. This may seem contradictory because PHAPI itself is a tumor suppressor and PP2A is also a tumor suppressor. However, no proper explanation has been discovered. The inhibitory function of PHAPI is probably dominant in the aspect of inducing apoptosis.

3.3. SET/I_2^{PP2A}. The oncogene SET and its truncated cytoplasmic form I_2^{PP2A} are also inhibitory proteins of PP2A. It is discovered that SET is fused with the nucleoporin NU214 (CAN), and it is associated with myeloid leukemogenesis and highly expressed in Wilms' tumors and BCR-ABL1-positive leukemia. Its overexpression predicts poor prognosis [41]. Elevated expression of SET has been linked to cell growth and transformation. SET can inhibit PP2A by forming an inhibitory protein complex with PP2A [39]. Besides, it can also form an inhibitory complex with nm23-H1 which can inhibit tumor metastasis [42].

Eichhorn et al. discovered that a novel apolipoprotein E-based peptide, COG112, can inhibit the interaction of SET with PP2AC, leading to increased PP2A activity. With increased PP2A activity, the p-Akt and c-Myc activity decreases. COG112 can also release SET from nm23-H1, thus, restoring the metastasis suppressor function of nm23-H1 [43]. Also, apolipoprotein E-mimetis peptides can bind to SET, therefore, restoring PP2A activity [44].

3.4. SETBP1. The SET binding protein (SETBP1) is a SET regulator, and it is fused in frame with a nucleoporin, NUP98. SETBP1 is overexpressed in 27.6% of acute myeloid leukemia at diagnosis and is associated with poor prognosis, particularly in elderly patients, as patients with SETBP1 overexpression had a significantly shorter overall survival and event-free survival in patients over 60 years. SETBP1 overexpression protects SET from protease cleavage which increases the amount of full-length SET protein and leads to the formation of a SETBP1-SET-PP2A complex. The SETBP1-SET-PP2A complex can inhibit PP2A and therefore promote the proliferation of leukemic cells [45, 46]. Piazza et al. found mutated SETBP1 (encoding a p.Gly870Ser alternation) to be a new oncogene present in atypical chronic myeloid leukemia as cells expressing this mutant exhibit higher amounts of SETBP1 and SET protein and lower PP2A activity [47].

4. PP2A-Activating Drugs

PP2A can be activated by various agents though direct or indirect interaction, like ceramide and FTY720. The general consequence of reactivation of PP2A in malignancies is apoptosis of cancer cells, and others may include cell proliferation inhibition and cell cycle arrest.

4.1. Ceramide. Ceramide has been shown to be a potent tumor suppressor which can trigger apoptosis and autophagy and limit cancer cell proliferation. The downstream of ceramide include many players, like PP2A, p38, JNK, Akt, and survivin. In ceramide-induced mitochondrial outer membrane permeabilization (MOMP), which is a key event in apoptotic signaling, the activation of PP2A is an important step to activate GSK-3β. Ceramide-activated PP2A can dephosphorylate GSK-3β at Ser9 through PI3K-Akt pathway [48]. In ceramide-induced cell cycle arrest, the accumulation of p27 is due to the activation of PP2A which leads to the inhibition of Akt [49].

4.2. FTY720. The immunosuppressant FTY720 is a sphingosine analogue that is approved for the treatment of relapsing multiple sclerosis. It can induce apoptosis in peripheral blood lymphocytes. Its anticancer function has been discovered in many kinds of malignancies, like breast cancer, leukemia, HCC, and prostate cancer, and so forth [50, 51]. However, the mechanism underlying the anticancer therapy varies. In HCC cells, the FTY720-induced apoptosis is mediated through the PKCδ signal. In leukemia cells, FTY720-direct mitochondrion-related apoptosis is mediated by FTY720-induced PP2A activation which is the outcome of FTY720 disrupting SET-PP2A interaction [51, 52].

Other PP2A-activating drugs, like forskolin, chloroethylnitrosourea, and vitamin E analogues, and so forth, are listed in Table 4. A PP2A-activating protein E1A is also described. It is reported to increase PP2A activity by upregulating PP2AC subunits, which results in the repression of Akt activation and the subsequent apoptosis [53].

5. Targeting PP2A in Digestive System Cancers

The PP2A alternations and PP2A inactivation have been described in many kinds of digestive system cancers, like colorectal cancer and HCC. Suppression of PP2A activity may serve a carcinogenesis role in alimentary system malignancies. PP2A subunits have been found to be mutated or deleted to some degree in various digestive system cancers. The subunit Aβ alternation has been detected in 15% of colorectal cancer, and it is related to its capacity of binding to a B and/or a C subunit, which leads to a decreased PP2A activity [13]. Besides the relatively low frequency of PP2A mutations and deletions, Tan et al. found that the epigenetic mechanism may play a dominant role in PP2A inactivation in colorectal cancer. They found that epigenetic silencing of PP2A regulatory B55β subunit can be detected in more than 90% of colorectal cancers [71]. The PP2A inhibitory protein CIP2A is increased in colorectal cancer and HCC, accompanied by impaired PP2A activity. So, in digestive system cancers, PP2A has its unique role in malignancy suppression and can be a target in anticancer therapy.

In colon cancers, researches reveal that resistance to antiangiogenesis therapy exists in CSLCs. It is the consequence of PP2A activity suppression in CSLCs in colon cancer cells. By suppressing PP2A, the p38 mitogen-activated protein kinase (MAPK) pathway is activated, which leads to the activation of (heat shock protein 27) Hsp27 and the subsequent antiapoptotic effect of Hsp27 [72]. PP2A inhibition in CSLCs of glioblastoma effectively controls the cell differentiation and/or death through modulating the Akt/mammalian TOR (mTOR)/GSK-3β pathway [73]. In colorectal cancer, PP2A inhibition is essential for the maintenance of CSLCs through the Akt Ser473/mTOR pathway [74]. So, the inhibition of PP2A confers CSLCs the characteristics of stem cells and is of significant importance to the initiation of cancers. The therapeutic reactivation of PP2A in CSLCs can be a possible anticancer treatment against drug resistance and recurrence. Wang et al. did find that by activating PP2A, silibinin can suppress the self-renewal of CLSCs and inhibit

Table 4: PP2A-activating drugs/protein.

Activating drugs/Proteins	Mechanism	Malignancies	Consequences	References
Ceramide	Activation of PP2A, leading to activation of GSK-3β or accumulation of p27	Prostate cancer	Apoptosis; Cell cycle arrest	[48, 49]
FTY720	Disrupt SET–PP2A interaction	Leukemia	Apoptosis	[51, 52]
Forskolin	Induces PP2A activity by increasing intracellular cAMP levels	Leukemia	Block proliferation; induce apoptosis	[62, 63]
Chloroethylnitrosourea	Augment methylation of PP2A, leading to Akt dephosphorylation	Melanoma	Reduce cell proliferation and survival	[64, 65]
Vitamin E analogues (i.e., α-tocopheryl succinate)	Reduce PKCα isotype (colon cancer) or inactivation of JNKs (prostate cancer) activity by increasing PP2A activity	Colon, prostate cancer	Apoptosis	[66, 67]
Carnosic acid	Downregulate AKT/IKK/NFκB by activation of PP2A	Prostate cancer	Apoptosis	[68]
Methylprednisolone	Upregulate PP2A B subunits	Myeloid leukemia	Cell differentiation	[69]
Dithiolethione	Increase PP2A concentration	Lung, breast cancer	Inhibit proliferation	[70]
E1A	Upregulate PP2A C subunits	Breast cancer	Apoptosis	[53]

sphere formation and tumor initiation in colorectal cancer [74].

As has been mentioned, ceramide is a potent tumor suppressor which can lead to tumor cell apoptosis and autophagy. By activating PP2A, ceramide can induce apoptosis and cell cycle arrest either by GSK-3β activation or p27 accumulation. Ceramide is a sphingolipid consisting of sphingosine, and sphingadienes (SDs) is another derivative from soy and other natural sphingolipids. SDs are reported to inhibit cell growth and tumorigenesis by inhibiting Wnt signaling through PP2A/Akt/GSK-3β pathway in colon cancer [75]. The chemopreventive effect of SDs relies on PP2A activation, which may serve as an upstream target of SDs in downregulating Wnt signaling. The activating mutations in Wnt signaling have been linked to the initiation of colorectal cancer [76]. However, the down-regulation of Wnt signaling by PP2A is not universal. Aspirin and mesalazine are found to be able to decrease Wnt/β-catenin in colorectal cell lines which make them possible chemopreventive agents. Aspirin and mesalazine treatment are both associated with phosphorylation of PP2A which is an inactive form of PP2A [77, 78]. Some other anti-colon-cancer agents like dihydroxyphenylethanol (DPE) are also reported to induce apoptosis or cell cycle arrest by activating PP2A [79].

In HCC, as mentioned above, CIP2A overexpression can be detected, and bortezomib can downregulate CIP2A and upregulate PP2A activity in HCC. The inhibition of CIP2A by bortezomib leads to tumor cell apoptosis and autophagy. HCC cells with high levels of CIP2A are more resistant to bortezomib treatment than those with low level of CIP2A. Erlotinib can also downregulate CIP2A which leads to apoptosis in HCC. A few erlotinib derivatives have been found as CIP2A-ablating agents in HCC cell line SK-Hep-1. The compounds N4-(3-Ethynylphenyl)-6,7-dimethoxy-N2-(4-phenoxyphenyl) quinazoline-2,4-diamine and N2-Benzyl-N4-(3-ethynylphenyl)-6,7-dimethoxyquinazoline-2, 4-diamine can induce HCC cell apoptosis by inhibiting CIP2A [80]. *Zanthoxylum avicennae* extracts (YBBEs) and diosmin can inhibit HCC cell line HA22T cell proliferation by activating PP2A [81, 82].

6. Conclusion

It has been almost 30 years since the first recognition that okadaic acid is a tumor promoter and targets PP2A [83], and emerging studies have made it solid that PP2A is a tumor suppressor and that its regulation can be a target for anticancer therapy. This review is mainly focused on the restoration and activation of PP2A in human malignancies by targeting PP2A inhibitory proteins or directly activating or upregulating PP2A. However, we should never neglect the controversy that exists that whether PP2A is a real tumor suppressor, because there are also ever-growing evidences against this fundamental hypothesis. For example, Zimmerman et al. found that the inactivation of PP2A, in particular, of the B56γ and B56δ subunits, is a crucial step in triggering apoptin-induced tumor-selective cell death [84]. Antitumor drugs like cantharidin control cell cycle and induce apoptosis by inhibiting PP2A [85], and cell viability inhibition and proapoptotic effect of cantharidin in PANC-1 pancreatic cancer is mediated by the PP2A/IκB kinase (IKKα)/IκBα/p65 NF-κB pathway [86]. Accordingly, PP2A-mediated anticancer therapy may include two opposed aspects, activation and inhibition, mainly depending on the cell types or the transforming agents. And despite the progress made in the field of targeting PP2A in anticancer

therapy, there is still a long way ahead to clinical application. Much effort is needed in the molecular mechanisms and medical translation of possible therapeutic agents targeting PP2A.

Conflict of Interests

The authors declare that they have no competing interests.

Author's Contribution

Weibo Chen participated in the study design and wrote the paper. Zhongxia Wang participated in the study design and edited the paper. Chunping Jiang participated in the study design and edited the paper. Yitao Ding conceived the study, participated in its design, and gave final approval of the version to be published. All authors have read and approved the final paper. Weibo Chen and Zhongxia Wang contributed equally to the work as cofirst authors.

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Research Article

Study of RNA Interference Targeting NET-1 Combination with Sorafenib for Hepatocellular Carcinoma Therapy *In Vitro* and *In Vivo*

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The aim of this study is to explore the inhibitory effects of RNA interference (RNAi) targeting NET-1 or combined with sorafenib on HCC *in vitro* and *in vivo* and the possible underlying mechanisms. The expressions of NET-1 mRNA and protein were detected by RT-QPCR and western blot. The ability of proliferation was determined by CCK-8 assay. Apoptosis was examined by flow cytometry (FCM). Abilities of migration and invasion were measured by scratch-wound assay and transwell assay. MHCC97H cells with stable transfection of NET-1shRNA were injected subcutaneously to prepare nude mice model of HCC and Caspase-3, Caspase-8, and Caspase-9 mRNAs of tumor tissues in different groups were examined. NET-1 mRNA and protein were reduced sharply in MHCC97H cells transfected with NET-1shRNA. The abilities of proliferation and migration were inhibited and apoptosis was promoted in either NET-1shRNA or sorafenib as compared with untreated cells *in vitro* and *in vivo* ($P < 0.05$). The mRNA levels of caspase-3, caspase-8, and caspase-9 of tumor tissues were reduced in different treatment groups compared with untreated group, particularly in combination group. ($P < 0.05$). The combination NET-1shRNA with sorafenib dramatically enhanced the effects of sorafenib antitumor ,which may involve in blocking ras signaling pathway and stimulating apoptotic pathways simultaneously.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide [1], with some certain geographic regions in developing countries where the incidence of HCC is 16–32 times higher than in developed countries. Gene therapy, a new and promising therapeutic strategy, has been used for many cancers including HCC. SiRNA-targeted silencing of the genes associated with tumor cell proliferation or metastasis, as one method of gene therapy, shows great potency on HCC treatment. New EST tetraspanin-1, also called NET-1(C4.8, Tspan-1, P503S), a member of the tetraspan superfamily (TM4SF) [2–4], seems to be rather expressed in most HCC than in normal adult liver tissues [5]. This attractive characteristic of tumor-specific expression could made NET-1 as potential therapeutic target for HCC.

Sorafenib, an oral multikinase inhibitor approved by the US Food and Drug Administration for the treatment of patients with advanced renal cell carcinoma (RCC) and those with refractory HCC. Recent years, *in vivo* and *in vitro* studies have shown that sorafenib could inhibit tumor growth and disrupts tumor microvasculature through antiproliferative, antiangiogenic, and/or proapoptotic effects. Sorafenib represents an important advance in the treatment of advanced HCC and is the first systemic therapy shown to prolong survival in advanced HCC. A number of trials examining the combined use of sorafenib plus chemotherapy agents (e.g., fluorouracil [6], gemcitabine [7], or capecitabine plus oxaliplatin [8]) or of sorafenib plus other molecularly targeted therapies (e.g., sirolimus [9]) are currently underway and are yielding promising results. However, studies about gene therapy using RNAi technology combination with sorafenib on HCC rarely have been reported. Therefore in the present study, we used NET-1shRNA combined with sorafenib to explore a novel strategy for treating HCC *in vivo* and *in vitro*.

Table 1: Primer sequences of NET-1, caspase 3, caspase 8, caspase 9, and GAPDH.

Primer	Sequence	Product (bp)
NET-1-F′	5′-GTGGCTTCACCAACTATACG-3′	191
NET-1-R′	5′-GACTGCATTAGTTCGGATGT-3′	
Caspase-3-F′	5′-AGAACTGGACTGTGGCATTGAG-3′	191
Caspase-3-R′	5′-GCTTGTCGGCATACTGTTTCAG-3′	
Caspase-8-F′	5′-CATCCAGTCACTTTGCCAGA-3′	128
Caspase-8-R′	5′-GCATCTGTTTCCCCATGTTT-3′	
Caspase-9-F′	5′-TTCCCAGGTTTTGTTTCCTG-3′	143
Caspase-9-R′	5′-CCTTTCACCGAAACAGCATT-3′	
GAPDH-F′	5′-TGATGACATCAAGAAGGTGGTGAAG-3′	240
GAPDH-R′	5′-TCCTTGGAGGCCATGTGGGCCAT-3′	

2. Methods

2.1. Cell Culture. Human HCC cell line MHCC97H was kindly provided by Liver Cancer Institute, Zhongshan Hospital, Shanghai. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) in humidified atmosphere containing 5% CO_2 at 37°C. The present experiments were divided into four groups including untreated group, sorafenib group, NET-1shRNA group and the combination NET-1shRNA with sorafenib group.

2.2. Transfection of Plasmids. pSilencer4.1-CMVneo-NET-1shRNA (NET-1shRNA) and pSilencer4.1-CMVneo-control shRNA (control shRNA) plasmids were designed and synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu, China). One day prior transfection, cells were cultured in medium without serum and antibiotics. After mixed gently and incubated for 20 minutes at room temperature, the transfection mixture of shRNA and Lipofectamine 2000 (Invitrogen) was added into culture plates. After 6 h, the transfection mixture was replaced by DMEM supplemented with 10% FBS. Cells were harvested at 48 h after transfection.

2.3. Real Time RT-QPCR. Total RNA was isolated with PicoPure RNA isolation Kit (Arcturus Bioscience, Mountain View, CA, USA) according to the manufacture's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control [10]. The PCR primers were designed by Premier Primer 5.0 software (Table 1). Primers (20 pmol/μL) were added into reaction buffer with a total volume of 25 μL together with template RNA 4 μL, Master Mix 12.5 μL, and SYBR Green I 0.5 μL. PCR was performed at the following conditions: 30 seconds at 94°C, 30 seconds at 62°C, and 40 seconds at 72°C for 50°C cycles. Real time RT-QPCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was performed using a MiQ machine (Bio-Rad Laboratories, Hercules, CA). The fluorescent signals were collected during the extension phase, Ct values of the sample were calculated, and NET-1 transcript levels were analyzed by 2-ΔΔCt method.

2.4. Western Blot Analysis. MHCC97H cells were harvested at 48 h after transfection. Cells were lysed with buffer containing 0.1 mol/L Tris-HCl (pH6.8), 4% SDS, 20% glycerine, 0.1% BPB, and 5% β-mercaptoethanol. The complex was heated in boiling water for 5 minutes. Proteins were separated by 10% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) at 350 mA for 2 h, which was later soaked for 2 h on a blocking solution (Tris-buffered saline containing 5% nonfat dry milk and 0.01% vol/vol Tween-20), and incubated for one hour at room temperature in the presence of Anti-NET-1 rabbit polyclonal antibody [11, 12], or anti-GAPDH mouse monoclonal antibody (Sigma, USA) used as internal control, then incubated at 4°C overnight. After incubation, the membrane was washed 3 times, and peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (ICN Laboratories, Irvine, CA; diluted 1 : 10,000) were added and incubated for an additional one hour. Reaction was visualized by the ECL chemiluminescence detection system (Pierce, USA) on radiographic films (Koda, USA). The molecular weight of NET-1 and GAPDH [13] were 76 kDa and 37 kDa, respectively. The results were analyzed using Image J software.

2.5. Cell Counting Kit-8 (CCK-8) Assays

2.5.1. IC50 Determination. Sorafenib was purchased from Bayer Pharmaceutical Corporation, dissolved in sterile DMSO, and stored frozen under light-protected conditions at −20°C. Stock solutions of sorafenib were diluted in DMEM culture medium, and equal aliquots were added to individual wells so that the final concentrations were 0, 3, 9, 18, 30, 60, and 100 μM. To determine the 50% inhibitory concentration (IC50) for sorafenib, the CCK-8 (Dojindo, Kumamoto, Japan) was used. MHCC97H cells were seeded at 5.0×10^3 cells/well in 100 μL of DMEM in 96-well microplates and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. After the cultures were incubated for 0 h, 24 h, 48 h, and 72 h, we exchanged fresh DMEM gently and 10 μL of CCK-8 solution was added to each well, and the plates were incubated for 2 h at 37°C. We measured the absorbance at 450 nm using an optical density (Microplate Reader 550; Bio-Rad, Tokyo, Japan) and calculated the IC50 concentration of sorafenib by the intersection of the plotted line (Figure 2). The IC50 of sorafenib was used in the growth, apoptosis, scratch-wound, and transwell assays.

2.5.2. Growth Assays. 5×10^3 cells in each group were seeded in 96-well plates and cultured for 0, 24, 48, and 72 h, the optical density at 450 nm wavelength was measured through an automated plate reader and then cell growth curves were drawn.

2.6. Flow Cytometry (FCM). Cellular apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit I (Clontech Laboratories Inc., USA) according to the manufacturer's protocol. Cells were seeded in 6-well plates at the density of 2×10^5/mL and harvested by trypsinization then washed with cold PBS, centrifuged at 1000 rpm, resuspended in 400 μL 1 × binding buffer, centrifuged again and removed supernatant. Cells were resuspended in 200 μL 1 × binding buffer and transferred to a sterile FCM glass tube. 3 μL Annexin V-FITC and 3 μL propidium iodide were added and then incubated in the dark at room temperature. Cells were analyzed by FCM (FACSCalibur, Becton-Dickinson, USA) at 488 nm. The distribution of cells was analyzed using CellQuest software (Becton-Dickinson) in the FCM within 1 hour of staining. Data from 10,000 cells was collected for each data file. Apoptotic cells were identified as Annexin V-FITC-positive and P-negative cells.

2.7. Scratch-Wound Assay. Cells were seeded at a density of 5×10^5 in 24 well plates and incubated overnight. The day after, the surfaces of the dishes were mildly scratched with a yellow P200 pipette tip (Fisher) and images were taken under a Carl Zeiss Axiovert 25 (Thornwood New York, USA) inverted microscope with the use of a Cannon PowerShot G9 digital camera using a 40X objective, which were totally observed continuously for 72 h.

2.8. Transwell Assay. Uncoated or Matrigel-coated transwells containing 8 μm pores were used for the assays (Costar, Corning, NY). Cells were seeded into the upper chamber in serum-free DMEM media. DMEM media containing 10% FBS was added to the lower chamber. Cells were fixed in 100% methanol 20 h later and stained with 0.2% concentration of crystal violet for 15 min at room temperature. Cells remaining on the upper side of the filter were removed with a cotton swab. The filters were then mounted onto cover slips and images were taken at 40X magnification. From these images, the number of migratory or invasive cells was counted.

2.9. G418 Selection. G418, an aminoglycoside antibiotic, is the most commonly antistable transfection reagents for screening in molecular genetic testing. Cells were seeded at the density of 2×10^5 cells/well in 24-well plates and grew overnight. The medium was replaced with complete medium without FBS. NET-1shRNA and control shRNA were transfected into MHCC97H cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was replaced with a fresh medium of bovine serum (150 mL/L) after 6 h transfection. One day later, the transfected cells were selected by G418 (400 μg/mL) (Huamei Biotechnology Company, Beijing, China) until positive clones were discovered after 14 days. The cells were cultured and finally selected by G418 (200 μg/mL) for a further 14 days. Single clones were selected to build a stable transfected cell line.

2.10. Animals and Establishment of Tumor Model

2.10.1. Animals. Nude BALB/c mice, female, 4–6 weeks old and weighing 18~20 g, were obtained from Shanghai Slac Laboratory Animal (China) and housed under pathogen-free conditions according to the recommendations of NIH guidelines for care and use of laboratory. This study has been approved by the Animal Ethical Committee of Nantong University.

2.10.2. Tumor Model. Nude mice were inoculated subcutaneously at the right anterior axilla with 1×10^7 stably transfected NET-1 MHCC97H cells in 200 μL PBS and at left anterior axilla with equal untreated cells, as autocontrol. The shortest axis (a) and the longest axis (b) of tumor were measured by caliper every day. The tumor volumes were calculated with the formula: volumes = $a^2 \times b \times \pi/6$. When the tumor volume reached 100 mm^3 at least, the tumor-bearing nude mice model was established successfully.

The experiments composed of 12 nude mice, which were randomly assigned to four experimental groups. The four experimental groups were as follows: untreated MHCC97H cells group, stably transfected NET-1shRNA MHCC97H cells group, sorafenib treating MHCC97H cells group, and sorafenib treating stably transfected NET-1shRNA MHCC97H cells group. In sorafenib treatment group, the mice were given 100 mg/kg sorafenib in 100 μL by peritoneal injection after tumor implantation established. All control mice received an equal volume of carrier solution by peritoneal injection.

At the 3 weeks of treatments, all treated mice were sacrificed, livers were excised and weighed, and one part was used for pathological examination and the others were stored at −80°C for detecting Caspase-3, Caspase-8, and Caspase-9 mRNA by real time RT-QPCR; the primer sequences were in Table 1.

2.11. Statistical Analysis. All experiments were performed in triplicate and the results were expressed as mean ± standard errors. All data were analyzed with SPSS13.0 statistical software using student's t-test. Two-tailed P values of <0.05 were considered statistically significant.

3. Results

3.1. NET-1 Expression in MHCC97H Cells. RT-QPCR and western blot analysis was performed to determine whether transfection with NET-1shRNA resulted in a reduction of the expressions of NET-1 mRNA and protein. As compared with control shRNA, there were 49% and 51% reduction of NET-1 mRNA and protein levels in cells transfected with NET-1shRNA, and no significant reduction of NET-1 expression was found in cells transfected with either control shRNA or untreated group (Figure 1).

To evaluate the proliferation of MHCC97H cells treated by NET-1shRNA, sorafenib, and combination of them, cells

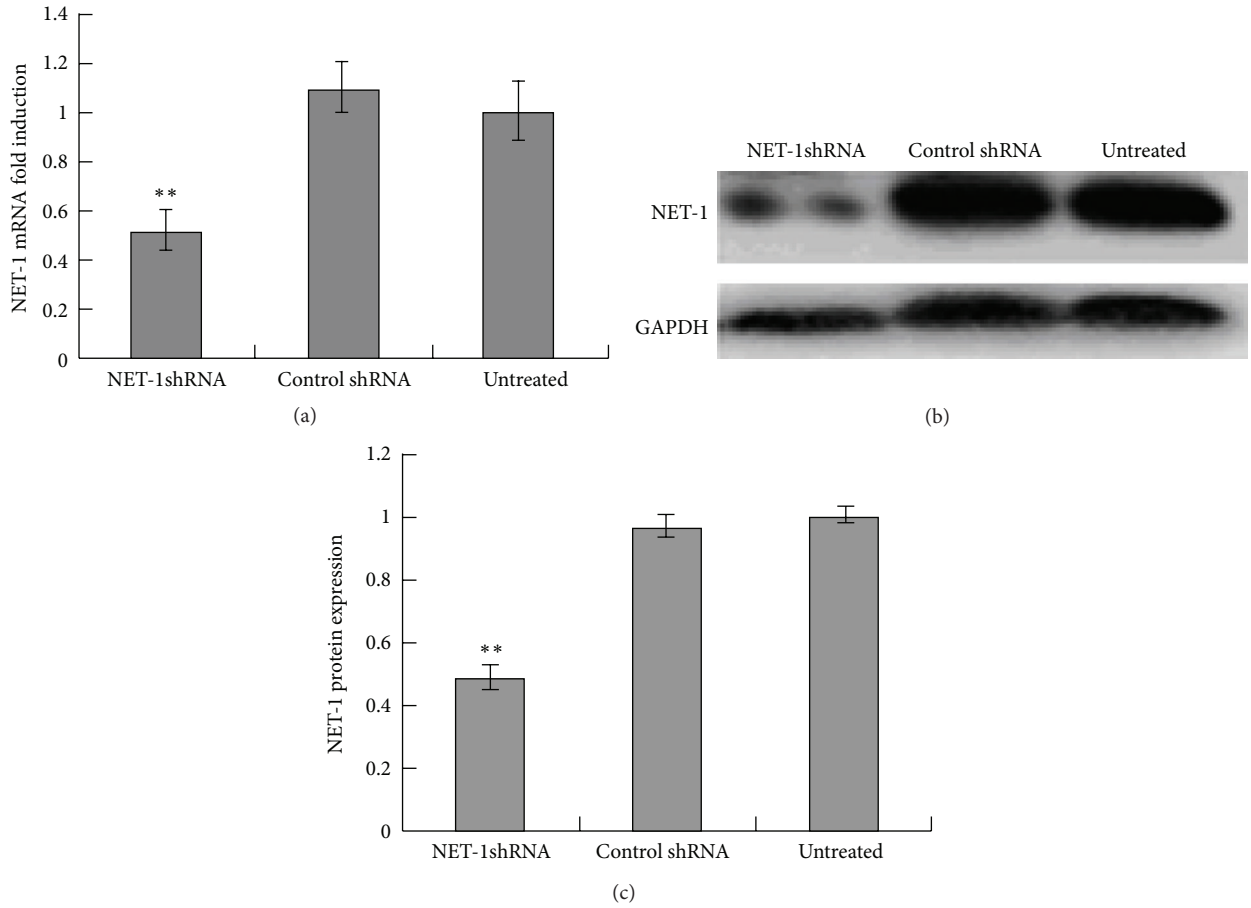

Figure 1: Inhibition of NET-1 mRNA and protein expression by NET-1shRNA in HCC cells line MHCC97H cells. (a) RT-QPCR showed NET-1shRNA resulted in 49% reduction of NET-1 mRNA levels, when compared with untreated group. (b) Western blot showed the intensities of NET-1 protein and GAPDH protein in these three groups. (c) Image J analyzed NET-1shRNA resulted in 51% reduction of NET-1 protein levels, when compared with untreated group ($^{**}P < 0.01$).

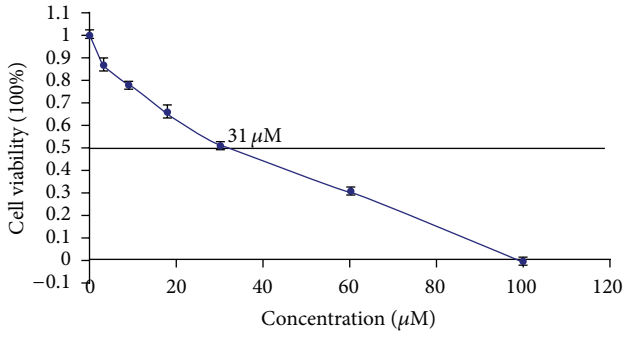

Figure 2: Dose-related cytotoxicity following 72 h exposured to sorafenib. The IC50 of sorafenib was indicated by the intersection of the plotted line. Sorafenib was 31 μM.

growth curves were obtained through CCK-8 assay. There was a significant reduction of proliferation in all treated cells, and especially in combination NET-1shRNA with sorafenib as compared with untreated cells (each $P < 0.01$). However, there was no significant difference in cell proliferation rates between sorafenib and NET-1shRNA groups (Figure 3).

3.2. Proliferation of MHCC97H Cells. MHCC97H cells in 96-well culture plates were exposed to different concentrations of sorafenib for 48 h. IC50 of sorafenib was calculated as 31 μM (Figure 2).

3.3. Apoptosis of MHCC97H Cells. FCM was used to examine the apoptosis of MHCC97H cells. The apoptosis rate rose up in all three treated groups compared with untreated group. Interestingly, combination NET-1shRNA with sorafenib notably advanced the apoptosis rate than either sorafenib or NET-1shRNA group ($P < 0.05$, resp.). But there was no difference between these two single groups (Figure 4).

3.4. Motility of MHCC97H Cells. Scratch-wound and trans-well chamber assays were conducted to evaluate the migration of MHCC97H cells in the presence of sorafenib, NET-1shRNA, and both of them. Under these conditions, Scratch-wound (Figure 5) and trans-well chamber (Figure 6) showed MHCC97H cells display different migrating potential compared to untreated cells, respectively. Interestingly, there was no disparity between NET-1shRNA group and sorafenib group. Cells were observed under an inverted microscope.

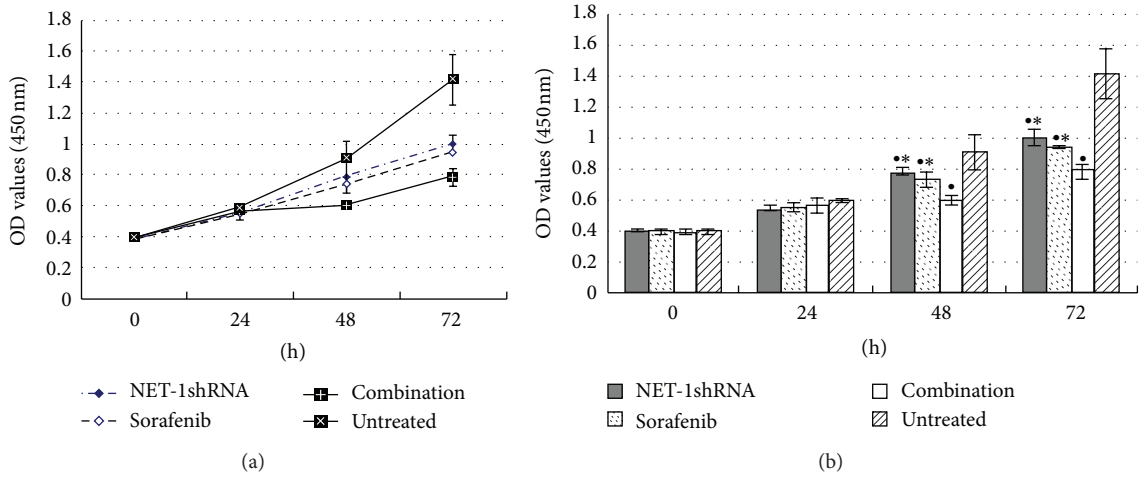

Figure 3: Growth curves showed the proliferation of MHCC97H cells until 72 h by CCK-8 assay. NET-1shRNA, sorafenib, and combination of NET-1shRNA with sorafenib resulted in 14.6%, 19.3%, and 33.3% reduction of cell proliferation at 48 h, in 46.5%, 50.6%, and 66.3% reduction of cell proliferation at 72 h. ($^{\bullet}P < 0.05$ compared with untreated group. $^{*}P < 0.05$ compared with combination group).

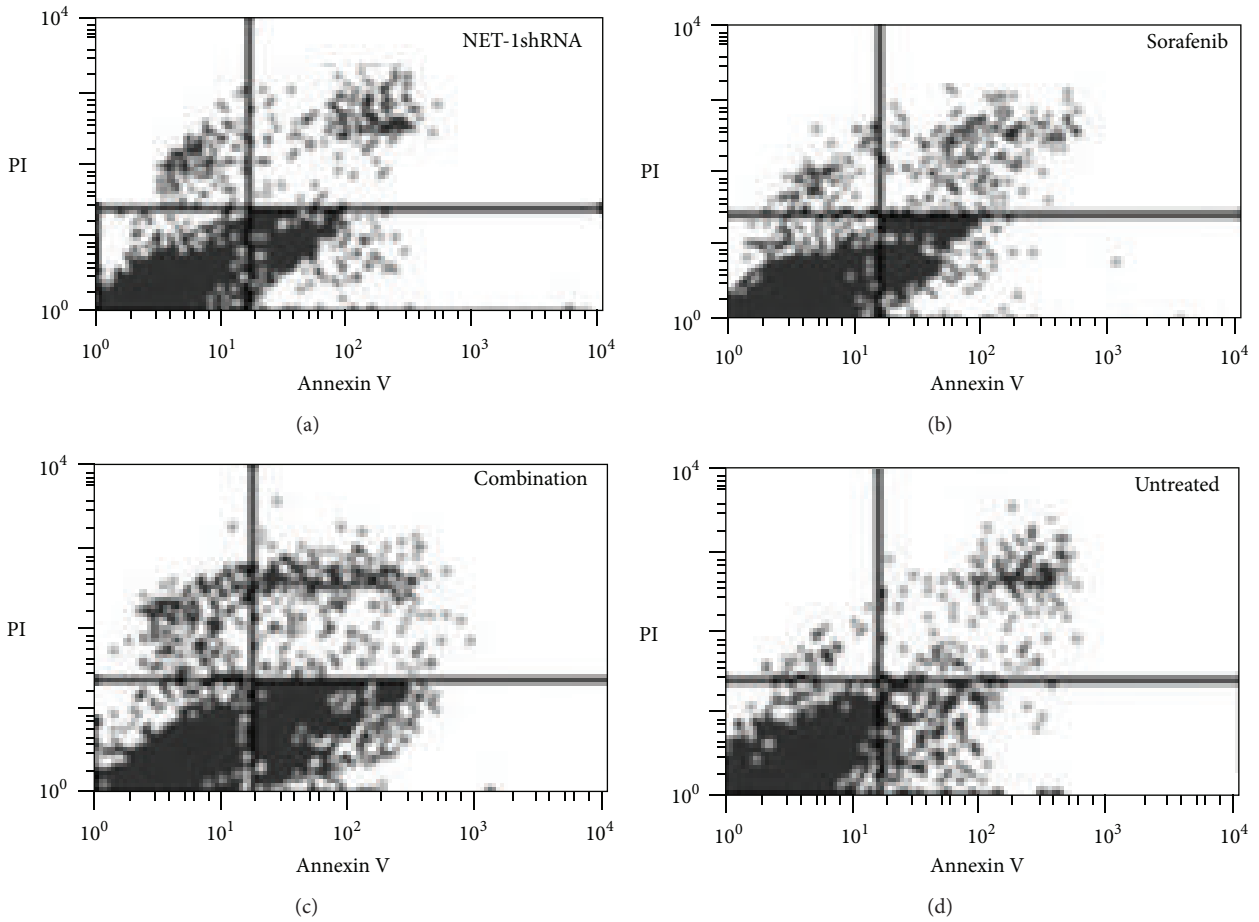

Figure 4: Percentage of apoptotic cells included both early- and late-stage apoptosis (AV+/PI− and AV+/PI+) was detected by FCM. The apoptosis rates of NET-1shRNA, sorafenib, combination, and untreated group were 24 ± 0.72%, 17.64 ± 0.46%, 51.34 ± 1.25%, and 6.3 ± 0.03%, respectively.

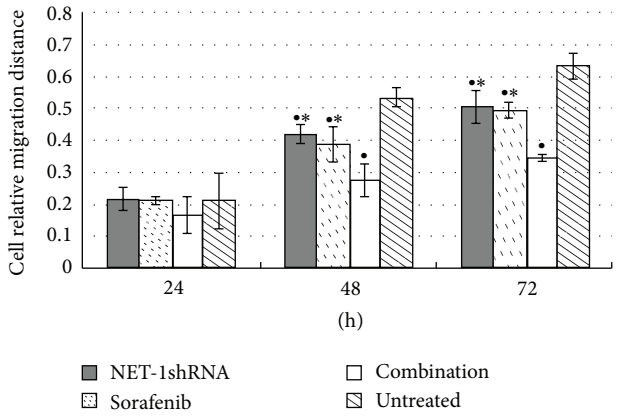

Figure 5: Cell relative migration distance at different time points in different groups. $^{\bullet}P < 0.05$ compared with untreated group. $^{*}P < 0.05$ compared with combination group.

3.5. Inhibition of HCC Model Growth by NET-1shRNA Alone or in Combination with Sorafenib. The tumor was used to mimic liver tumor growth in mice. The average tumor size presented statistical differences in these four groups. There was smaller tumor size in all treated groups than that in untreated group ($P < 0.05$, resp.). Notably, combination group in the inhibiting effect was superior to each single group ($P < 0.05$, resp.). However, no statistical difference was found between NET-1shRNA group and sorafenib group (Table 2).

To further investigate the mechanism about inhibiting tumor growth, we detected the mRNA levels of caspase-3, caspase-8, and caspase-9 of the tumor tissues from these four groups. The results of real time RT-QPCR showed caspase 3 in NET-1shRNA, sorafenib, and combination groups significantly increased, respectively, when compared with untreated group (each $P < 0.05$). Because Caspase 8 mRNA levels significantly increased in NET-1shRNA and combination groups, Caspase 9 mRNA levels significantly increased in sorafenib and combination groups, respectively, when compared with untreated group (each $P < 0.05$), so that increasing expressions of caspase-3, Caspase-8, and Caspase-9 mRNA in combination group were higher than that in each single group ($P < 0.05$, resp.) (Figure 7).

Table 2: Comparing HCC growth treated by NET-1shRNA alone and in combination with sorafenib.

Groups	MHCC97H (cm^3)
NET-1shRNA	$1.354 \pm 0.042^{\bullet\star}$
Sorafenib	$1.201 \pm 0.152^{\bullet\star}$
Combination	$0.558 \pm 0.018^{\bullet}$
Untreated	3.459 ± 0.121
Empty vector	3.279 ± 0.084

The average tumor size in each group was $1.354 \pm 0.042\,cm^3$, $1.201 \pm 0.152\,cm^3$, $0.558 \pm 0.018\,cm^3$, and $3.459 \pm 0.121\,cm^3$, respectively, ($^{\bullet}P < 0.05$ compared with untreated group. $^{\star}P < 0.05$ compared with combination group).

4. Discussion

NET-1 as a novel tumor relative gene is over-expressed in many malignant tumors including the breast, uterine cervix, colon, esophagus, liver, lung, ovary, pancreas, prostate, gastrointestinal, and skin [10, 14–28]. Recent studies have shown that NET-1 is involved in a variety of processes such as oncogenesis, cell cycle control, apoptosis, and migration. Wollscheid et al. [17] found that NET-1 gene expression correlated to cell proliferation and may be used as a marker for cervical cancer prognosis.

RNAi technology is a powerful approach to silence mammalian gene expression for studies of gene function and has the potential for gene therapy. Synthetic siRNA can trigger RNAi response in mammalian cells and induce specific inhibition of gene expression. FDA approval has been granted for an investigational new drug license to test the use of expressed RNA sequences against HBV [29], bringing a promise for RNAi treatment on HCC. The specific shRNA used in our experiments has been exploited by Chen et al. and confirmed effective in previous research [10]. In the present study, NET-1 mRNA and protein expressions were significantly reduced by 49% and 51%, respectively, using NET-1shRNA.

NET-1 was proved to be associated with neoplastic cell proliferation [20]. Our data indicated an inhibition of tumor cell proliferation by 46.5% after single-agent sorafenib treatment, and 50.6% after NET-1shRNA transfection, respectively. Combined treatment with sorafenib plus NET-1shRNA significantly enhanced the antiproliferative effect of sorafenib on HCC *in vitro*. Furthermore, combined treatment also inhibited tumor volume of the tumor-bearing nude mice model more assertively than treated with a single agent *in vivo*. NET-1 belongs to the member of the guanine nucleotide exchange factor (GEF) family, the latter helps small G protein mutual transition from GDP to GTP, thus activating Ras and Rho. So our findings implied that NET-1 may promote tumor proliferation and growth through activating Ras signal transduction pathway, which mediates tumor cell proliferation, differentiation, and survival. The antitumor effect of sorafenib plus downregulation NET-1 by RNAi may achieve a synergistic effect on the inhibition of cell growth by inhibiting several relevant pathways.

NET-1 has 30% identity with metastasis-associated tetraspans (e.g., Co-029 and Talla-1) [2]. Tetraspanins were implicated in regulation of signaling, motility, migration, and invasiveness [30]. Huang et al. showed a MK-induced NET-1 pathway contributing to migration/invasiveness of human head and neck squamous cell carcinoma cells then found RNAi silencing of NET-1 dramatically decreased MK-induced expression of MMP-2, which demonstrated the role of NET-1(TSPAN1) as one of various signaling components [31]. Our results revealed that migration and infiltration of MHCC97H cell were dramatically reduced after transfection of NET-1shRNA. Combination therapy with sorafenib and NET-1shRNA amplified the inhibition effects of sorafenib on tumor migration/invasiveness from 57.71% to 74.46%, compared with the untreated. Meanwhile, Sorafenib has been classified as a vascular endothelial growth factor receptor (VEGFR) inhibitor, which blocks tumor angiogenesis by decreasing microvessel density and circulating levels of

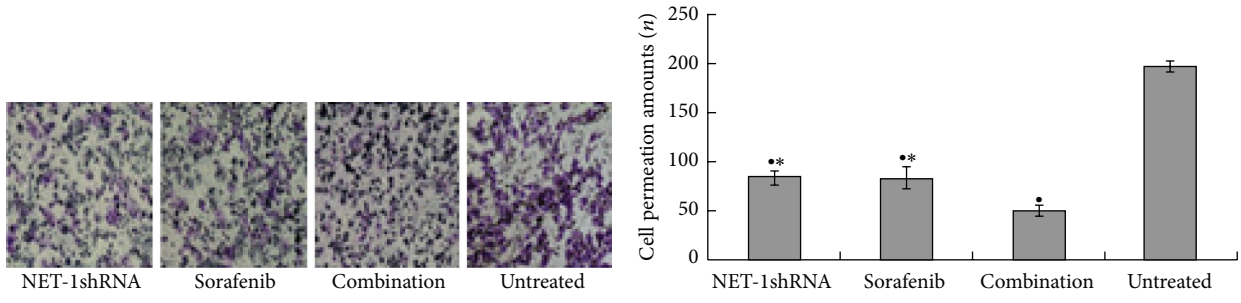

Figure 6: Motility of MHCC97H cells was inhibited by NET-1shRNA and sorafenib. (a) Trans-well chamber showed the permeation amounts of MHCC97H cells after 48 h of NET-1shRNA transfection or sorafenib treatment. (b) Trans-well chamber assay suggested the permeation amounts of MHCC97H cells in NET-1shRNA, sorafenib, and combination NET-1shRNA with sorafenib and untreated cells groups were 83.67 ± 6.03, 83.33 ± 10.01, 50.33 ± 4.73, and 197.00 ± 6.00, respectively. ($^{\bullet}$ represents $P < 0.05$ compared with untreated group. * represents $P < 0.05$ compared with combination group).

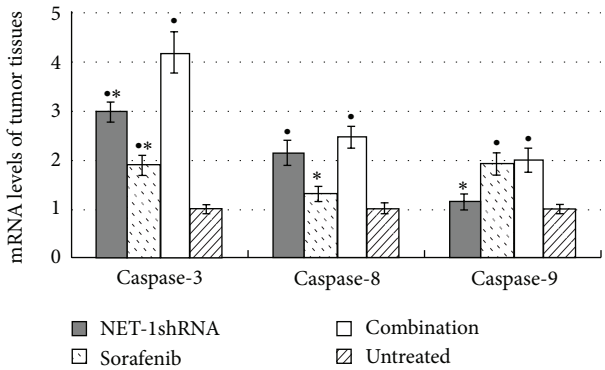

Figure 7: The expression mRNA levels of caspase 3, caspase 8, and caspase 9 of tumor tissues in NET-1shRNA, sorafenib, combination, and untreated groups. RT-QPCR showed that in NET-1shRNA, sorafenib, and combination groups caspase 3 mRNA levels increased by 2.99, 1.89, and 4.18 times, Caspase 8 mRNA levels increased by 2.14, 1.31 and 2.47 times, and Caspase 9 mRNA levels increased by 1.15, 1.91, and 1.99 times, compared with untreated group, respectively. ($^{\bullet}P < 0.05$ compared with untreated group, $^{*}P < 0.05$ compared with combination group).

VEGF. VEGF is one of the most potent angiogenic factors. MMP2 is a marker associated with angiogenesis as well as metastatic invasion [32]. Several studies demonstrated that the intensity of angiogenesis in HCC correlated with the risk of vascular invasion, metastasis, and patient prognosis [33, 34]. Therefore, these two agents (sorafenib and NET-1shRNA) in combination may boost the effect of each single agent.

Sorafenib has recently been found to induce apoptosis in several human cancer lines. Although the mechanism through which sorafenib induces apoptosis has not been fully elucidated, Yu et al. found sorafenib induced apoptosis by downregulating myeloid cell leukemia-1(Mcl-1) [35], which was presumed to be associated with the release of cytochrome c from mitochondria into the cytosol, caspase activation, and apoptotic cell death. *In vitro* apoptosis assay proved this hypothesis: the apoptosis rate of sorafenib treated group was higher than untreated cells. Moreover, our results revealed that caspase 3 and caspase 9 mRNA levels changed significantly after sorafenib treatment. Interestingly, from the results, we could find that caspase 3 mRNA level increased dramatically and the caspase 8 mRNA increased in NET-1shRNA and combination groups. The increase of caspase 8 mRNA led by NET-1shRNA perhaps hinting that NET-1 was probably an apoptosis related gene associated with the death receptor pathway mediated by membrane receptor. In addition, *in vitro* results demonstrated that reduction of NET-1 by RNAi may also induce apoptosis. Under our deduction, it is easy to explain why caspase 3 mRNA levels increased greatly in combination group. As for the relationship between NET-1 and caspase 8, we still need to explore the mechanism of NET-1shRNA induced apoptosis through other effective methods.

Taken together, in this study NET-1shRNA has obviously suppressed proliferation, survival, and migration/invasiveness of HCC both *in vitro* and *in vivo*. NET-1shRNA manifested inhibitory effect on HCC as well as sorafenib. Furthermore, combination of sorafenib plus NET-1shRNA showed better antitumor effect than single-agent treatment. These findings would bring a new therapy for HCC.

Acknowledgments

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Research Article

Significance of Glutathione Peroxidase 1 and Caudal-Related Homeodomain Transcription Factor in Human Gastric Adenocarcinoma

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Aim. To investigate the expressions of glutathione peroxidase 1 (GPX1) and caudal-related homeodomain transcription factor (CDX2) in GAC and their correlation with clinicopathological features and tumor cell proliferation. *Methods*. The expressions of GPX1, CDX2, and Ki67 were immunohistochemically evaluated in 172 GAC specimens. The association of GPX1 and CDX2 with patient's clinicopathological features and Ki67 positive rate was analyzed statistically. *Results*. In 172 cases of GAC, the expression of GPX1 was weaker than that in adjacent normal mucosa, and the expression of CDX2 was higher than that in adjacent normal mucosa. High expression GPX1 strong-expression was associated with differentiation, Lauren type, WHO type and extensive lymph node metastasis of GAC. High expression of CDX2 was associated with differentiation, Lauren type, WHO type, extensive lymph node metastasis, and TNM of GAC. Survival curves showed that expressions of GPX1 and CDX2 were factors of good outcome ($P = .03$ and .02, resp.). According to multivariate analysis, only lymph node metastasis, TNM stage, and CDX2 expression were independently associated with survival. In addition, a strong association of GPX1 expression was noted with Ki67 and CDX2. *Conclusions*. The expression of GPX1 and CDX2 may play a role in the carcinogenesis, differentiation, and progression of GAC, and CDX2 may be an independent prognostic factor.

1. Introduction

Gastric adenocarcinoma (GAC) is one of the most common fatal malignancies in the world. The incidence varies considerably between geographical areas, with a higher incidence in China and other Asian countries than in Western Europe and the United States [1]. Stomach cancer was the fourth most common cause of cancer-related death from cancer in Europe in 2012 [2]. In China, gastric cancer was the second most common cancer in 2009 [3]. Patients with gastric cancer that is limited to the mucosa and submucosa have an excellent prognosis, with a 5-year survival rate of over 90% after surgery [4]. In contrast, the prognosis for patients with advanced cancer is generally poorer and less predictable. At present, therapeutic decisions are based on clinicopathological parameters, including age, tumor node metastasis (TNM) stage, and histological grade. Although useful, these factors often fail to differentiate more aggressive tumor types from less aggressive tumor types [5]. As GAC is a markedly heterogeneous disease with respect to histological features and biological characteristics especially in the advanced stages, previous studies have shown that its biological behavior and prognosis could be significantly different among the patients with the same stage, histological type, or differentiation grade.

Searching for special markers that are closely related to bionomic characteristics and outcome is still one of the major foci of research on GAC, although a number of biomarkers have already been found to be involved in the development and progression of GAC.

The antioxidant enzyme glutathione peroxidase 1 (GPX1) is part of the enzymatic antioxidant defense in normal cells. GPX1 catalyses the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduced glutathione, thereby protecting cells against oxidative damage [6–8]. Decreased activity of these antioxidant enzymes may increase oxidative stress and damage to several biomolecules, including DNA, which may initiate or promote neoplastic transformation in affected tissues [9]. The loss of GPX1 expression was associated with aggressiveness and poor survival in patients with gastric cancer [10].

The homeobox transcription factor caudal-related homeodomain transcription factor (CDX2) plays a crucial role in intestinal cell fate specification, and it is a critical determinant of intestinal homeostasis both during development and throughout adult life. Furthermore, there are substantial pieces of evidence supporting the crucial role of CDX2 in carcinogenesis of the digestive tract. CDX2 was shown to inhibit cell growth and migration *in vitro*, as well as the dissemination of colon tumor cells *in vivo* [11]. CDX2 reduction increases the progression of chemically induced colorectal cancers [12]. Conversely, under certain pathological conditions, CDX2 becomes abnormally expressed in other organs of the digestive tract other than the intestine, such as the esophagus [13] and stomach [14, 15].

In this study, we immunohistochemically examined the expressions of GPX1, CDX2, and Ki67 in 172 samples of human gastric adenocarcinomas. The association of the expression of GPX1 and CDX2 with various histopathological features was assessed, and the relationship between GPX1 and CDX2 was evaluated.

2. Material and Methods

2.1. Patient Data. Tumor specimens were obtained from 172 patients (108 males and 64 females; age range 24 to 82 years) who underwent surgery for gastric adenocarcinomas from November 2005 to April 2008. None of the patients had received prior chemotherapy or radiotherapy. All patients provided written informed consent. Clinical and pathological records and slides were available for all cases. HE-stained slides of gastric adenocarcinomas were reviewed. Histopathological examination indicated that 20 cases were well differentiated, 42 were moderately differentiated, and 110 were poorly differentiated. Among the 172 cases, 64 GAC samples were of intestinal type according to Lauren-type classification, 70 were of diffuse type, and 38 were of mixed type, respectively. According to the World Health Organization (WHO) histological classification, 142 patients were diagnosed as tubular type, 12 patients were diagnosed as mucinous type, 4 patients were diagnosed as of papillary type, and 14 patients were diagnosed as of signet ring cell type. A total of 35 cases had no lymph node involvement, 42 had one lymph node involved, and 95 had more than one lymph node involved. According to TNM classification, there were 10 cases at stage I, 18 at stage II, 80 at stage III, and 64 at stage IV.

2.2. Processing of Specimens and Immunohistochemistry. For protein expression analysis, 172 gastric adenocarcinomas and 16 adjacent normal mucosa (ANM) tissues samples were detected by using immunohistochemical staining. All of the tissue samples were histologically verified. Sections (4 μm) of tissue blocks were transferred to an adhesive-coated slide. A 3-step immunoperoxidase technique using streptavidin-peroxidase (S-P) was employed for GPX1, CDX2, and Ki67 detection. All sections were routinely deparaffinized and rehydrated, and then the sections were rinsed in phosphate-buffered saline (PBS, pH = 7.4), and subsequently were treated for antigen retrieve (10 min, microwave oven, 10 mM EDTA, pH 8.0). After cooling at room temperature for 20 min, the sections were rinsed in PBS and immersed in 3% H_2O_2 for 15 min to block the endogenous enzymes. After being rinsed in PBS, the sections were incubated with normal goat serum at 37°C for 15 min to block nonspecific antibodies. The primary antibody was a polyclonal goat antiserum for GPX1 (Rabbit mAb, Eipt Mics, USA,1:50), CDX2 (Rabbit mAb, EPR2764Y, Maxim, China, ready for use), and Ki67 (Mouse mAb, MIB-1, Maxim, China, ready for use). After being incubated with primary antibody at 37°C for 60 min, the sections were rinsed in PBS, incubated with biotinylated secondary antibody (SP kit, Maxim, China), and rinsed in PBS again. After interaction with streptavidin-HRP (SP kit, Maxim, China) a rinse in PBS, the sections were visualized by reaction with 3,3′-diaminobenzidine and counterstained with hematoxylin. Normal gastric and intestinal mucosa were used as positive controls for GPX1 and CDX2, respectively. A lymph node was used as a positive control for Ki67, and normal goat serum and PBS substitution for the primary antibody were used as negative controls.

2.3. Scoring of the Results. All of the staining results were evaluated by two independent researchers (JH and HL) without the knowledge of the clinicopathological data. If the two investigators assigned different scores, consensus was obtained in all of the cases after discussion.

2.3.1. GPX1. For the staining of GPX1, a positive stain was defined as brown stain observed in the cytoplasm. Tissues with no evidence of staining or only rare scattered positive cells less than 3% were recorded as negative. The immunohistochemical results were evaluated for the intensity and frequency of staining. The intensity of staining was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The frequency was graded from 0 to 4 by the percentage of positive cells as follows: grade 0, <3%; grade 1, 3–25%; grade 2, 25–50%; grade 3, 50–75%; and grade 4, more than 75%. The index score was the product of multiplication of the intensity and frequency grades, which was then classified using a 4-point scale: index score 0 = product of 0, index score 1 = products 1 and 2, index score 2 = products 3 and 4, and index score 3 = products 6 through 12 [16].

2.3.2. CDX2. For the staining of CDX2 and Ki67, a positive stain was defined as brown stain observed in the nuclei. The whole area of slide was evaluated and positive CDX2 and positive Ki67 were defined by more than 10% positive cancer cells [17].

2.4. Statistical Analysis. All of the statistical analyses were performed with SPSS 8.0 software for Windows. The Kruskal-Wallis rank-sum test was used to compare GPX1 expression with clinicopathological factors, and the Wilcoxon rank-sum test was used to compare CDX2 expression with clinicopathological factors and both factors with Ki67. The survival analysis of patients was determined using the Kaplan-Meier method and Cox regression, and statistical evaluation was performed using the log rank test. $P < .05$ was considered statistically significant.

3. Results

3.1. Expression of GPX1 in GAC and Adjacent Normal Mucosa (ANM) Tissues. Out of 172 gastric adenocarcinomas, 1 case (0.6%) had an index score of 3 (the case scored as a 3 was merged with those cases scored as a 2, statistically), 35 cases (20.3%) had index scores of 2, 96 cases (55.8%) had index scores of 1, and 40 cases (23.3%) had index scores of 0 (Figures 1(a)–1(d)). The total positive rate of GPX1 in all 172 cases of GAC was 76.7% (132/172). All 16 of the ANM tissues samples were scored as a 3. The expression of GPX1 in ANM tissues was significantly stronger than that in GAC ($Z = -7.170$, $P = .000$, $r = .664$). The higher positive rate of GPX1 was associated with better differentiation ($\chi^2 = 53.401$, $P = .000$, $r = .541$). The total expressive rate of GPX1 was 100% (20/20) in the well-differentiated group, 92.9% (39/42) in the moderately differentiated group and 66.3% (73/110) in the poorly differentiated group. The positive rate of GPX1 was associated with Lauren type ($\chi^2 = 22.389$, $P = .000$, $r = .308$). In 64 cases of intestinal type, the total positive rate was 92.2% (59/64), which was higher than that in the diffuse (61.4%, 43/70) and mixed types (78.9%, 30/38) (Table 1). The expression of GPX1 was associated with WHO type. The expression of GPX1 in the tubular type was stronger than that in the other types ($\chi^2 = 28.081$, $P = .000$, $r = .396$). In 142 cases of tubular type, 36 cases were scored as 2, and 23 were scored as 1. In 30 cases of other types, no cases were scored as 2, and 13 were scored as 1. The expression of GPX1 was negatively associated with lymph node involvement ($\chi^2 = 9.710$, $P = .008$, $r = -.230$). Out of 35 cases with no lymph node involvement, 12 cases were scored as 2, 19 were scored as 1, and 4 were scored as 0. Out of 42 cases with one lymph node involved, 10 cases were scored as 2, 25 were scored as 1, and 7 were scored as 0. Out of 95 cases with more than one lymph node involved, 14 cases were scored as 2, 52 were scored as 1, and 29 were scored as 0. The expression GPX1 was not associated with age, sex, or TNM stage ($P > .05$) (Table 1).

3.2. Expression of CDX2 in GAC and Adjacent Normal Mucosa (ANM) Tissues. Out of 172 gastric adenocarcinomas, 102 cases (59.3%) demonstrated positive expression of CDX2 and 70 cases (40.7%) were negative (Figures 1(e)–1(h)). Out of 16 ANM tissues samples, none was positive for CDX2. The positive rate for CDX2 in GAC was significantly higher than that in ANM tissues ($Z = -4.540$, $P = .000$, $r = .332$) (Figure 1). The higher positive rate of CDX2 was associated with better differentiation ($Z = -5.336$, $P = .000$, $r = .398$). The expressive rate of CDX2 was 95% in the well-differentiated group, 81.0% in the moderately differentiated group, and 44.5% in the poorly differentiated group. The positive rate of CDX2 was associated with Lauren type ($Z = -3.962$, $P = .000$, $r = .291$). In 64 cases of intestinal type, 52 cases were positive and the positive rate was 81.3%, which was higher than that in the diffuse and mixed types (50/108, 46.3%). The positive rate of CDX2 was negatively associated with lymph node involved ($Z = -5.027$, $P = .000$, $r = -.335$). In 34 cases of no lymph node involved, 29 cases (85.3%) were positive for CDX2. In 42 cases with one lymph node involved, 33 cases (78.6%) were positive. In 96 cases with more than one lymph node involvement, 40 cases (41.7%) were positive (Table 1). The positive rate of CDX2 was negatively associated with the TNM stage ($Z = -2.243$, $P = .025$, $r = -.177$). In 28 cases of stages I and II, 24 cases (85.7) were positive. In 144 cases of stage III and IV, 78 cases (54.2%) were positive. The expression CDX2 was not associated with age, sex, or WHO type ($P > .05$) (Table 1).

3.3. Prognostic Implication of GPX1 and CDX2 Expression in GAC. The median survival time was 26 months for patients with GPX1 expression scored as 2, 17 months for patients scored as 1, and 11 months for patients scored as 0. The Kaplan-Meier method was used to analyze the association between the total survival rate and the expression of GPX1. The patients with stronger expression of GPX1 showed a significantly higher survival rate than patients with weaker expression of GPX1 ($P = .030$) (Figure 2). The median survival time of patients with positive CDX2 expression was 23 months, and that of the patients with negative CDX2 expression was 12 months. The Kaplan-Meier method was used to analyze the association between the total survival rate and the expression of CDX2. The patients with a higher positive rate of CDX2 showed a significantly higher survival rate than patients with a lower positive rate ($P = .021$) (Figure 2). Based on the Cox regression analysis of the 172 patients, CDX2 expression, lymph node metastasis, and TNM stage seemed to be independent prognostic indicators ($P = .026$, .011, and .001, resp., Table 2).

3.4. Correlation of GPX1 and CDX2 Expression with Ki67. The expression of GPX1 was negatively associated with Ki67. Cases of GPX1 scored as 2 had a higher Ki67 positive rate than those scored as 1 and 0 ($Z = -3.843$, $P = .000$, and $r = -.294$) (Figures 1(j)–1(l)). The positive rate of CDX2 was not associated with the positive rate of Ki67 ($Z = -1.434$, $P = .152$) (Table 3).

3.5. Correlation of GPX1 with CDX2 Expression in GAC. The expression of GPX1 was positively associated with CDX2 in GAC ($Z = -4.754$, $P = .000$, and $r = .363$, Table 4).

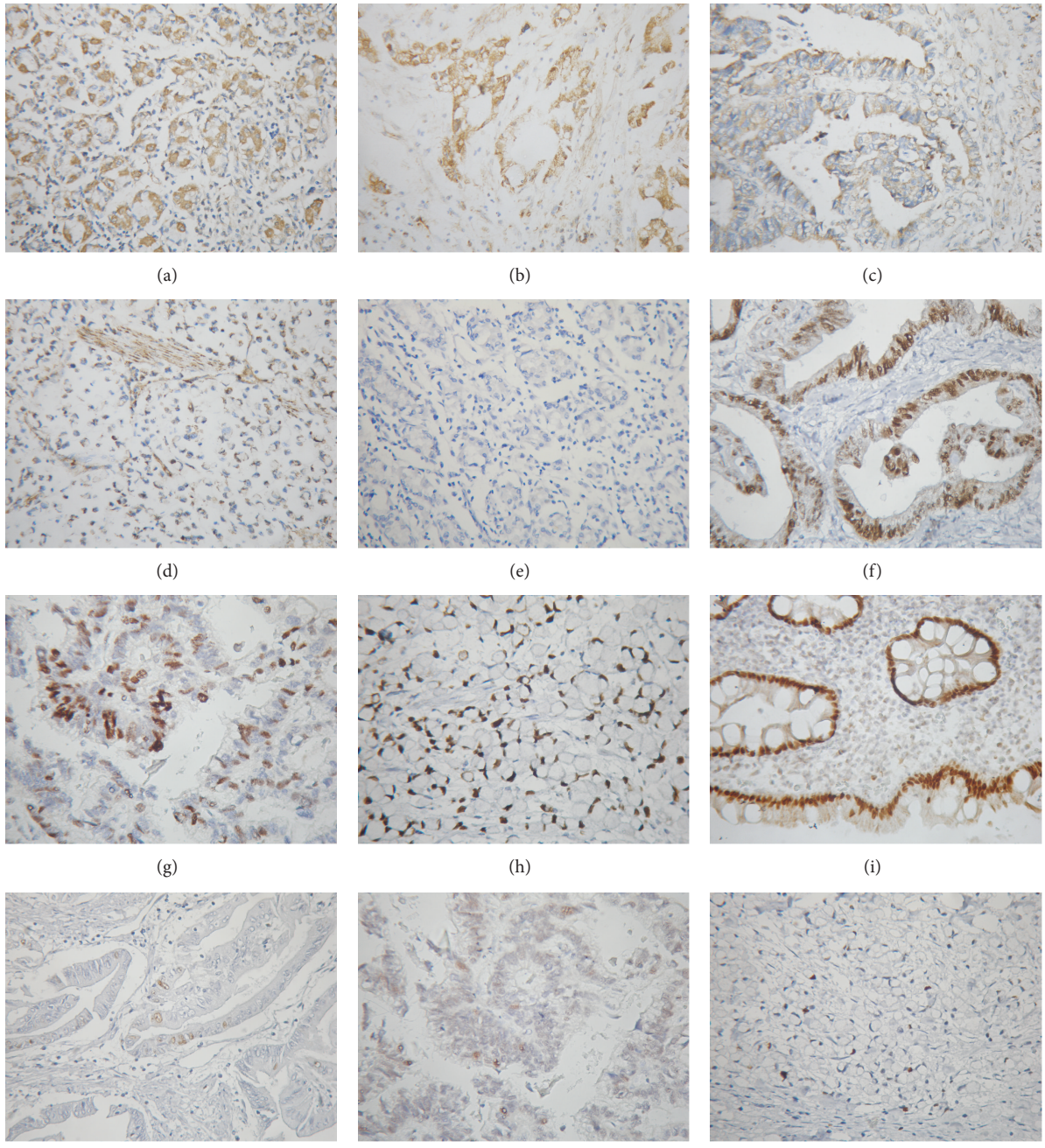

Figure 1: Immunohistochemical staining in GAC and ANM tissue. (a) GPX1 expression in ANM tissue (score 3). (b) GPX1 expression in well-differentiated GAC (score 3). (c) GPX1 expression in well-differentiated GAC (score 2). (d) GPX1 expression in signet-ring cell GAC (score 0). (e) CDX2 expression in ANM tissue (negative). ((f) and (g)) CDX2 expression in well-differentiated GAC (positive). (h) CDX2 expression in signet-ring cell GAC (negative). (i) CDX2 expression in normal colon mucosa (positive). ((j) and (k)) Ki67 expression in well-differentiated GAC (negative). (l) Ki67 expression in signet-ring cell GAC (negative). Original magnification: ×200.

4. Discussion

Selenium has been shown to be effective in reducing carcinogenesis in animal model systems, and human studies supporting a protective role of this element have been reported. Selenoproteins whose ultimate levels can be influenced by selenium suggest a mechanism for how this element reduces cancer incidence. Normal levels of selenium that are present during homeostasis upregulate endogenous antioxidant defenses by increasing GPX activity [18]. GPX1 plays an important role in the protection of cells from oxidative stress by reducing hydrogen peroxide with glutathione. The GPX-1 gene has been found to be a frequent LOH locus in cancers of the lung, breast, and ovary [19]. In this study, we

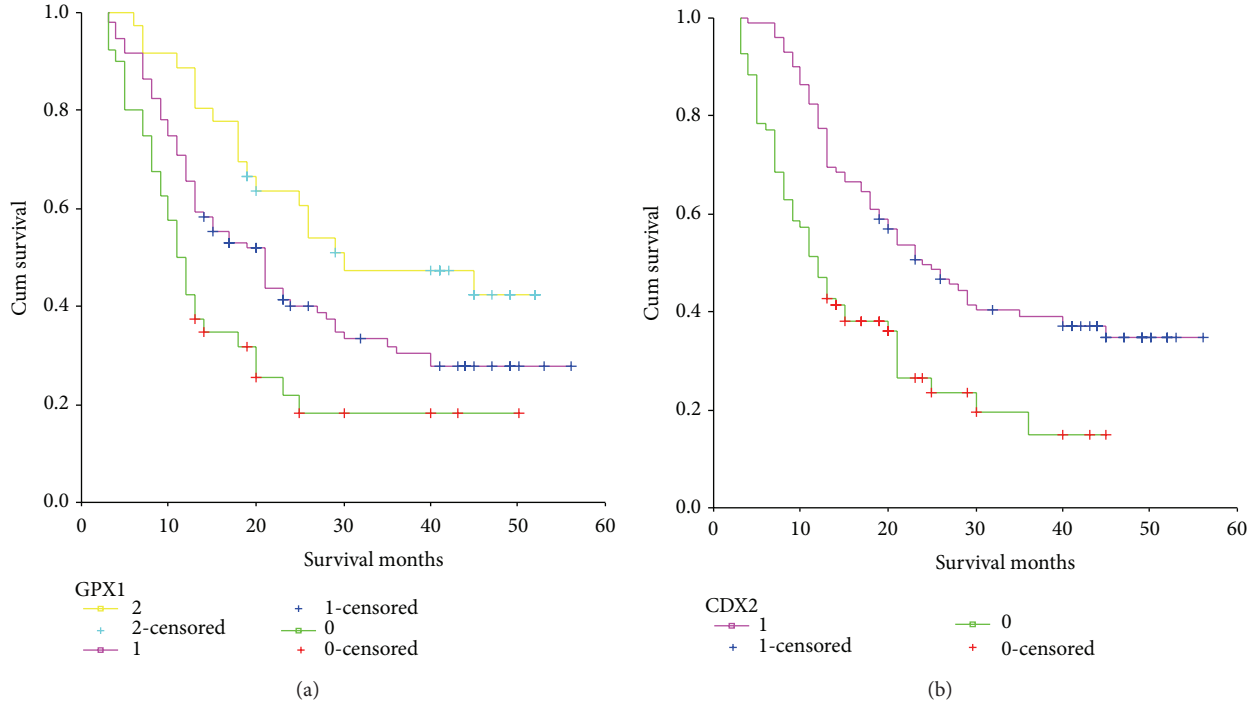

Figure 2: Survival curve of gastric cancer patients (a) GPX1 high expression group compared with GPX1 weak expression group and (b) CDX2 positive expression group compared with CDX2 negative expression group ($P = .030$ and .021).

found a significant loss of GPX1 expression in GAC compared with ANM, which demonstrated that the loss of GPX1 was involved in the carcinogenesis of GAC. Reduced levels of GPX-1 may increase the risk or promote the development of cancer [20]. Similar results were found in this study. The loss of cytosolic expression of GPX1 was associated with poor differentiation and extensive lymph node involvement. Expression of GPX1 in the group of poorly differentiated carcinomas and the group with more than one lymph node involved was significantly lower than that in the groups with well or moderately differentiated carcinoma and no lymph node involvement. Moreover, stronger expression of GPX1 was associated with a favorable survival. The loss of GPX1 expression was associated with aggressiveness and poor outcome in patients with GAC. However, nuclear expression of GPX1 may have different effects. In hepatocellular carcinoma (HCC), selenium-binding protein 1 (SBP1) and GPX1 formed nuclear bodies and colocalized under oxidative stress. A decrease of SBP1 was linked with increased GPX1 activity, and it correlated with vascular invasion and a poor outcome [21]. GPX1 expression in different locations of tumor cells may have a different impact on tumor development, which warrants further investigation. GPX1 expression was associated with the Laurence type and WHO classification of tumor subtypes, but the mechanism is unclear. Accounting for the relationship of intestinal-type GAC with survival, the association of GPX1 and Lauren type may be incidental. GPX1 was found to be significantly increased and survivin was reduced following resveratrol treatment in non-small-cell lung carcinoma cells, which suggested that GPX1 might be involved in the inhibition of tumor cell proliferation [22]. In this study, stronger expression of GPX1 was found to be association with low Ki67 positive rate. This result suggested that the loss of GPX1 increased tumor cell proliferation.

CDX2 was found to be intensively involved in intestinal metaplastic differentiation [23]. CDX2 may stimulate intestinal proliferation and differentiation by transcriptional activation of intestine-specific proteins (MUC2, sucrase-isomaltase, and carbonic anhydrase I). Aberrant expression of CDX2 is prominent in intestinal-type gastric adenocarcinoma and CDX-2 may therefore play an important role in gastric carcinogenesis, especially in the intestinal type of GAC [24]. In this study, we found that CDX2 was not expressed in normal gastric mucosa, but it was expressed in 59.3% of GAC, which showed abnormal upregulation in GAC. Correa [25] proposed that human gastric carcinogenesis is a multistep process that progresses in the following order: chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and gastric cancer. Although CDX2 was reported not to be associated with the intestinal metaplastic subtype [26], CDX2 was found to be intensively involved in intestinal metaplastic differentiation [23]. We demonstrated that CDX2 expression was associated with the Lauren type and WHO type of GAC in this study. The data demonstrated that CDX2 was expressed more in well differentiated, intestinal type and tubular type than that in the other types of GAC. These results suggest that CDX2 may play a role in the differentiation of the tumor cells after carcinogenesis. In this study, CDX2 expression was decreased more in the group with more than one lymph node involved and in the stage III/IV (TNM) group than in the

Table 1: Relationship of GPX1 and CDX2 expression with clinicopathological factors of GAC.

Factors	n	GPX1 score			CDX2	
		0	1	2	−	+
Age						
<50	59	17	35	7	26	33
≥50	113	23	61	29	44	69
Sex						
Male	108	28	55	25	39	69
Female	64	12	41	11	31	33
Differentiation						
Well	20		5	15*	1	19*
Moderate	42	3	25	14	8	34
Poor	110	37	66	7	61	49
Lauren type						
Intestinal	64	5	31	28*	12	52*
Diffuse	70	27	40	3	38	32
Mixed	38	8	25	5	20	18
WHO type						
Tubular	142	23	83	36*	54	88
Mucinous	12	5	7		6	6
Papillary	4		4			4
Signet-ring cell	14	12	2		10	4
Lymph node involvement (number)						
No	35	4	19	12*	6	29*
One	42	7	25	10	9	33
>1	95	29	52	14	55	40
TNM stage						
I	10	1	7	2	3	7*
II	18		10	8	1	17
III	80	20	46	14	36	44
IV	64	19	33	12	30	34

*$P < .05$.

Table 2: Association of various factors with overall survival by logistic regression.

Variable	B	S.E.	Wald	df	Sig	R	Exp(B)
Age	.2276	.2233	1.0388	1	.3081	.0000	1.2556
Sex	−.1806	.2182	.6848	1	.4079	.0000	.8348
Differentiation	.0216	.2534	.0073	1	.9321	.0000	1.0218
Lauren type	−.2320	.1691	1.8834	1	.1700	.0000	.7929
WHO type	.0235	.1917	.0150	1	.9026	.0000	1.0237
LN involved	.2983	.1154	6.6818	1	.0097	.0662	1.3475
TNM stage	.3888	.1525	6.4967	1	.0108	.0649	1.4752
Ki67	.4773	.4485	1.1323	1	.2873	.0000	1.6117
GPX1	−.3468	.1837	3.5629	1	.0591	−.0383	.7069
CDX2	−.5168	.2321	4.9561	1	.0260	−.0526	.5964

groups with no lymph node involvement/one lymph node involved and the stage I/II (TNM) group. We also found that CDX2 expression was associated with a favourable outcome. Similarly, CDX2 was reported as a prognostic factor that acted as a marker of good outcome in patients with gastric cancer [27]. Based on our results, CDX2 transcriptional factor might act as a tumour suppressor [28, 29].

In our study, we demonstrated that the expression of GPX1 was correlated positively with the expression of CDX2 in GAC, but the mechanism is not clear.

Table 3: Association of GPX1 and CDX2 with Ki67 in GAC.

	n	GPX1 score		CDX2	
		0-1	2	−	+
Ki67					
−	47	28	19*	15	32
+	125	108	17	55	70

* $P = .000$.

Table 4: Association of GPX1 with CDX2.

	GPX1 score		
	0	1	2
CDX2			
−	28	36	6
+	12	60	30

$P = .000$.

GPX is involved in resistance to Adriamycin [30]. Moreover, CDX2 activates the *Multidrug Resistance 1* gene in gastrointestinal cancers [31]. We speculate that GPX1 and CDX2 either cooperate or simply coexist in their roles in drug resistance in GAC, which warrants further investigation.

Conflict of Interests

No potential conflict of interests was disclosed in this paper.

Authors' Contribution

Jing-jing Han and De-rong Xie have contributed equally to this work.

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Review Article

Indirect Comparison Showed Survival Benefit from Adjuvant Chemoradiotherapy in Completely Resected Gastric Cancer with D2 Lymphadenectomy

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Background. Little data on directly comparing chemoradiotherapy with observation has yet been published in the setting of adjuvant therapy for resected gastric cancer who underwent D2 lymphadenectomy. The present indirect comparison aims to provide more evidence on comparing the two approaches. *Methods*. We conducted a systematic review of randomized controlled trials, extracted time-to-event data using Tierney methods (when not reported), and performed indirect comparison to obtain the relative hazards of adjuvant chemoradiotherapy to observation on overall and disease-free survival. *Results*. Seven randomized controlled trials were identified. Three trials compared adjuvant chemoradiotherapy with adjuvant chemotherapy, and 4 trials compared adjuvant chemotherapy with observation. Using indirect comparison, the relative hazards of adjuvant chemoradiotherapy to observation were 0.43 (95% CI: 0.33–0.55) in disease-free survival and 0.52 (95% CI: 0.38–0.71) in overall survival for completely resected gastric cancer with D2 lymphadenectomy. *Conclusions*. Postoperative chemoradiotherapy can prolong survival and decrease recurrence in patients with resected gastric cancer who underwent D2 gastrectomy. Molecular biomarker might be a promising direction in the prediction of clinical outcome to postoperative chemoradiotherapy, which warranted further study.

1. Introduction

Gastric cancer is the third leading cause of cancer-related death among men and the fifth among women in the worldwide [1]. The primary curative treatment of gastric carcinoma is surgical resection [2]. Complete resection with adequate margins is widely considered as a standard goal, whereas the extent of lymph node dissection remains controversial. Irrespective of the surgical procedure used for the treatment of gastric cancer, the effectiveness of surgical resection is poor; about 60% eventually have local relapse or distant metastases after curative resection [3]. The high rate of relapse or distant metastases after resection make it important to consider adjuvant treatment for patients with resected gastric cancer.

The INT-0116 trial [4, 5], the largest phase III trial comparing chemoradiotherapy versus observation, shows that adjuvant chemoradiotherapy prolonged overall survival (OS) and relapse-free survival (RFS). In this trial, 10% of the patients underwent D2 dissection, suggesting that chemoradiotherapy might be only compensating for inadequate surgery. Therefore, the role of chemoradiation therapy after D2 dissection has been questioned. Two retrospective studies demonstrated that adjuvant chemoradiotherapy was well tolerated with acceptable toxicities and reasonable tumor control for patients with D2 gastrectomy [6, 7]. Another retrospective study does not demonstrate that adjuvant chemoradiotherapy reduce relapse and impact on survival [8]. There is no RCT comparing adjuvant chemoradiotherapy with observation D2-dissected gastric cancer. In view of the paucity of data, we attempted to answer this question using the method of adjusted indirect comparison.

2. Methods

2.1. Literature Search. A systematic review of eligible RCTs was performed by searching the electronic databases, which

consist of Cochrane Central Register of Controlled Trials, Medline, EMBASE, ISI Web of Knowledge, ASCO abstracts, and ESMO abstracts. The deadline of this search was June 30, 2013. The keywords were used for search in electronic databases as follows: "gastric cancer," "Stomach Neoplasms," "chemoradiotherapy," "chemoradiation," "Chemotherapy," "D2", and "Combined Modality Therapy." The search was limited to RCTs in English language. The reference lists of articles were identified, and relevant meta-analysis were searched manually to find other relevant articles.

2.2. Trial Selection and Quality Assessment. All RCTs that compared chemotherapy with observation or compared chemoradiotherapy with chemotherapy in adjuvant therapy for resected gastric cancer were included in the present study. If the same population appeared in other publications, the article that provided the most complete follow-up data on survival was selected. Methodological quality of the trials was assessed using a validated scale (range, 0 to 5) applied to items that influence intervention efficacy. The scale consists of items pertaining to randomization, masking, dropouts, and withdrawals, which is reported by Jadad et al. [9]. Trial was regarded as high quality trial and had high external and internal validities if it was scored by more than 3 points.

2.3. Data Extraction. Two primary reviewers assessed all abstracts that were identified from the above-mentioned sources. Both reviewers independently selected potentially eligible abstracts according to inclusion criteria. If one of the reviewers concluded an abstract that might be eligible, the complete article was retrieved and reviewed in detail by both reviewers. Disagreements were resolved by consensus or by the third reviewer. Hazard ratio (HR) and 95% confidence interval (95% CI) for OS and DFS were requested. Where published, HR and 95% CI were extracted directly from the original article. Where HR and 95% CI were not reported, they were calculated from published summary statistics or survival curve using Tierney et al. method [10]. The following variables were extracted from each trial if available: first author's name, publication year, country of origin, treatment regimen, total numbers of patients, percentage of different stages, percentage of T3 and T4 stage, percentage of lymph node positive, HR and 95% CI for OS and DFS, and median follow-up time.

2.4. Brief Introduction of Adjusted Indirect Comparison. Suppose that interventions A and C were directly compared in a RCT, and another trial compared intervention B with intervention C. To compare intervention A with intervention B, a method of adjusted indirect comparison can be used to realize it [11]. Briefly, the log hazard ratio (log HR) of the adjusted indirect comparison for intervention A versus B was estimated by $\log \mathrm{HR}_{\mathrm{AB}} = \log \mathrm{HR}_{\mathrm{AC}} - \log \mathrm{HR}_{\mathrm{BC}}$, and its standard error for the log HR was $\mathrm{SE}(\log \mathrm{HR}_{\mathrm{AB}}) = \sqrt{\mathrm{SE}(\log \mathrm{HR}_{\mathrm{AC}})^2 + \mathrm{SE}(\log \mathrm{HR}_{\mathrm{BC}})^2}$.

Where $\log \mathrm{HR}_{\mathrm{AC}}$ was the log HR for the direct comparison of intervention A versus C and $\log \mathrm{HR}_{\mathrm{BC}}$ were the log HR for the direct comparison of intervention B versus C. $\mathrm{SE}(\log \mathrm{HR}_{\mathrm{AC}})$ was the standard error of the log HR for the direct comparison of intervention A versus C and $\mathrm{SE}(\log \mathrm{HR}_{\mathrm{BC}})$ was the standard error of the log HR for the direct comparison of intervention B versus C. The strong underlying assumption in this adjusted indirect comparison method is that the relative efficacy of an intervention is consistent in patients included in different trials. That is, $\log \mathrm{HR}_{\mathrm{AC}}$ observed in trials comparing A versus C is assumed to be $\log \mathrm{HR}_{\mathrm{BC}}$ that would have been observed in those trials comparing B versus C and vice versa.

2.5. Statistical Analysis. To combine the results of individual trial's HR for comparing chemoradiotherapy with chemotherapy or comparing observation with chemotherapy, direct meta-analysis was used. Heterogeneity assumption was checked by a chi-square-based Q-test and also expressed as I^2. A P value of more than 0.10 for the Q-test and I^2 of less than 50% indicated a lack of heterogeneity across the trials. If P-value of heterogeneity test was more than 0.1 and I^2 was less than 50%, fixed effects model was performed and random effects model was used vice versa.

Adjusted indirect comparison was used to evaluate the relative efficacy of chemoradiotherapy to observation. The primary end point was OS, and the secondary end point was DFS. Treatment effect size was calculated by HR and 95% CI. Due to the adjusted indirect comparison using the fixed effect model which tended to underestimate standard errors of pooled estimates, random effect model was used for the quantitative pooling in the adjusted indirect comparison. A HR value of less than 1 stands for favoring chemoradiotherapy arm and a HR value of more than 1 stands for favoring chemotherapy arm. All CIs had a two-sided probability coverage of 95%. A statistical test with a P value less than 0.05 was considered significant, and all P values were two-sided.

All analyses were performed strictly with RevMan software (version 5.2, Cochrane).

3. Results

3.1. Trial Flow, Characteristics, and Quality Appraisal. Figure 1 was the flow chart of RCTs selection for this study. Seven trials were identified at last [12–18]. Three trials compared chemoradiotherapy with chemotherapy. Four trials directly compared observation with chemotherapy. Six out of 7 trials were conducted in Asian countries, but only one trial was from European country. Almost all patients (>95%) underwent curative gastrectomy with D2 lymphadenectomy. Table 1 showed important baseline characteristics and Jadad scores of selected trials.

3.2. Adjusted Indirect Comparison. First, we use a method of meta-analysis to combine the pooled result for chemoradiotherapy versus chemotherapy and observation versus

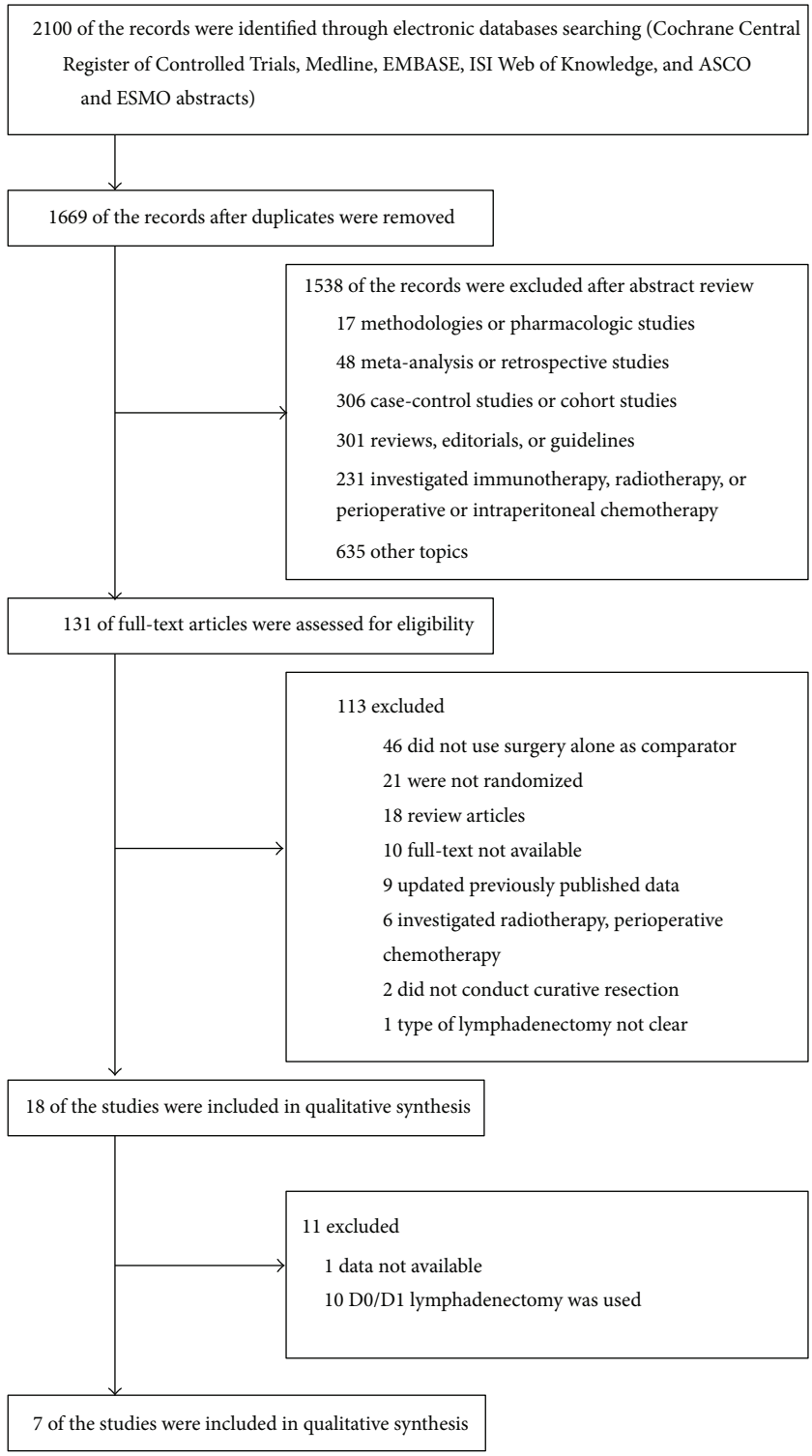

Figure 1: Flow chart of randomized controlled trials selection.

chemotherapy. The pooled HR and 95% CI were 0.72 (0.59–0.89) in DFS and 0.79 (0.61–1.03) in OS for chemoradiotherapy versus chemotherapy. The pooled HR and 95% CI were 1.68 (1.46–1.93) in DFS and 1.52 (1.30–1.79) in OS for observation versus chemotherapy.

Second, adjusted indirect comparison was computed for estimating the relative efficacy of adjuvant chemoradiotherapy to observation. Compared with observation, chemoradiotherapy significantly improved DFS and OS for patients with D2-resected gastric cancer. The pooled HR and 95%

Table 1: Characteristics of selected RCTs.

References	Countries	Regimens	*N*	I/II/III/IV (%)	T3/4 (%)	N+ (%)	DFS HR (95% CI)	OS HR (95% CI)	Median (range) Follow-up (month)	Jadad score
Chemoradiotherapy versus chemotherapy										
Lee et al. 2012 [12]	Korea	XP/XRT/XP	230	21.3/36.5/30.8/11.3	NR	88.3	0.73 (0.52–1.04)	—	53.2 (36.9–77.3)	3
		XP	228	21.9/37.7/28.6/11.8	NR	84.6				
Zhu et al. 2012 [13]	China	RT/FL	168	10.8/19.4/55.4/14.5	100	84.9	0.74 (0.56–0.97)	0.80 (0.6–1.06)	42.5	3
		FL	165	9.1/18.2/58.2/14.5	100	86.7				
Kim et al. 2012 [14]	Korea	RT/FL	46	0/0/73.9/26.1	69.5	100	0.62 (0.33–1.14)	0.76 (0.39–1.48)	86.7 (60.3–116.5)	3
		FL	44	0/0/75/25	56.8	95.4				
Observation versus chemotherapy										
Cirera et al. 1999 [15]	Spain	MMC/tegafur	76	0/0/100/0	92	80	1.82 (1.18, 2.79)	1.67 (1.08, 2.57)	37 (3–122)	4
		Observation	72	0/0/100/0	89	97				
Nakajima et al. 2007 [16]	Japan	Uracil-tegafur	93	0/100/0/0	0	100	2.27 (1.28, 1.04)	2.08 (1.13, 3.85)	74.4	5
		Observation	95	0/100/0/0	0	100				
Sasako et al. 2011 [17]	Japan	S-1	529	0.2/49.9/42.3/7.6	45.1	90.4	1.54 (1.27, 1.86)	1.49 (1.20, 1.85)	60	3
		Observation	530	0/53.2/40.2/6.6	46.1	87.9				
Bang et al. 2012 [18]	Korea	XELOX	520	1/49/51/0	45	91	1.79 (1.40, 2.28)	1.39 (1.00, 1.93)	34.2 (25.4–41.7)	3
		Observation	515	0/51/49/0	45	89				

RCTs: randomized controlled trials; *N*: number of patients; I/II/III/IV: cancer stage; IV refers to T4N1-3M0 and T1-3N3M0; T3/4: percentage of T3 and T4 stage; N+: percentage of node positive; DFS: disease-free survival; OS: overall survival; HR: hazard ratio; 95% CI: 95% confidence interval.
NR: not report.
XP: capecitabine + cisplatin; XRT: radiotherapy with capecitabine; RT: radiotherapy; FL: fluorouracil plus leucovorin; MMC: mitomycin; XELOX: oxaliplatin + capecitabine.

Table 2: Indirect comparison on the efficacy of chemoradiotherapy versus observation for resected gastric cancer after D2 lymphadenectomy.

CRT versus Obs	Number of trials in comparison	Hazard ratio (95% CI)	*P* value
DFS	7	0.43 (0.33, 0.55)	0.00
	6*	0.43 (0.34, 0.56)	0.00
OS	6	0.52 (0.38, 0.71)	0.00
	5*	0.53 (0.38, 0.72)	0.00

CRT: chemoradiotherapy, Obs: observation, 95% CI: 95% confidence interval, DFS: disease-free survival, and OS: overall survival. *Subgroup analysis after omitting the trial from Spain.

CI were 0.43 (0.33–0.55) in DFS and 0.52 (0.38–0.71) in OS, respectively. Table 2 summarized those estimates of indirect comparison for D2-resected gastric cancer.

3.3. Subgroup Analyses. To explore the potential influence on survival benefit by geographic difference, we reevaluated the pooled HR for observation to chemotherapy by omitting the trial from Spain and did further indirect comparison of chemoradiotherapy to observation. The pooled HR for observation to chemotherapy was 1.66 (1.44–1.92) in DFS and 1.50 (1.26–1.78) in OS, respectively. Accordingly, the pooled HR for indirect comparison of chemoradiotherapy to observation was 0.43 (0.34–0.56) in DFS and 0.53 (0.38–0.72) in OS, respectively (Table 2).

4. Discussion

In the last decade, postoperative chemoradiotherapy has become the preferred strategy for resected gastric cancer in the United States because the INT-0116 trial suggested that postoperative chemoradiotherapy had a survival advantage over observation. However, adoption of this regimen has been somewhat tempered in Asian countries. The main reason was the inadequate node dissection (only 10% had a D2 dissection) in INT-0116. Recently, gastrectomy with D2 lymphadenectomy becomes the standard treatment for curable gastric cancer in Eastern Asia. Thus, the efficacy of adjuvant chemoradiotherapy should be established in patients with D2-resected gastric cancer.

To evaluate the relative efficacy of treatment approaches, the most reliable evidence comes from head-to-head RCTs. However, there is usually no direct randomized evidence or no sufficient direct randomized evidence. In this situation, adjusted indirect comparison of different interventions can be used to give an alternative estimation. It is reported that results of adjusted indirect comparison usually, but not always, agree with those of head to head randomized trials [19]. Due to insufficient direct evidence, we used an adjusted indirect comparison method to estimate the efficacy of adjuvant chemoradiotherapy to observation in completely resected gastric cancer. Overall, our data demonstrated strong benefit from adjuvant chemoradiotherapy in patients with D2-resected gastric cancer.

A Singapore retrospective study reports the clinical outcomes of 67 patients who were mostly treated with D2 node dissection and received adjuvant chemoradiotherapy as per INT-0116. The 3-year overall survival, disease-free survival, and local control are 60.6%, 54.1%, and 84.3%, respectively. Of the 30 patients who relapsed, 5 (17%) have isolated locoregional recurrences only. This retrospective study shows reasonable tumor control benefit from adjuvant chemoradiotherapy [6]. Comparable results were also showed in a Korean retrospective observational study with over 500 cases after D2 gastrectomy [7]. On the other hand, 3-year overall survival, disease-free survival, and local control in chemotherapy arm are 80.1%, 72.2%, and 75% in ACTS-GC trial, respectively [17]. Similarly, 3-year disease-free survival and local control in chemotherapy arm are 74% and 82% in CLASSIC trial, respectively [18]. Although simple horizontal comparison is unscientific, it seemed that chemoradiotherapy arm of two retrospective studies did not show an advantage over chemotherapy arm of ACTS-GC and CLASSIC trials. To date, 3 head-to-head trials compare chemoradiotherapy versus chemotherapy for those patients without positive results reported [12–14]. Furthermore, pooled analysis of these 3 trials also does not demonstrate that chemoradiotherapy has any survival advantage over chemotherapy [20].

The reason that chemoradiotherapy did not have any survival advantage over chemotherapy in D2-resected gastric cancer was not well understood. It has been reported that the sites of treatment failure after surgical treatment were mainly locoregional in the tumor bed in Western countries [21]. In contrast, in Asian countries, the sites of treatment failure were mainly distant metastasis [20]. The discrepancy is mainly due to a high percentage of diffuse-type histology gastric cancer in Asian population, which accounted for 50% at least [22]. Diffuse gastric cancer is characterized by decreased intracellular adhesion as a result of E-cadherin mutation and/or hypermethylation and is prone to early metastasis. Therefore, chemoradiotherapy does not appear to confer a benefit to diffuse gastric cancer [23].

There are studies to explore the role of molecular biomarkers in predicting clinical outcome to chemoradiotherapy. A study evaluates the potential association of xeroderma pigmentosum group D (XPD) codon 751 variant with outcome after chemoradiotherapy in 44 patients with resected gastric cancer. It indicates that 75% of relapse patients show Lys/Lys genotype more frequently ($P = 0.042$). The Lys polymorphism is an independent predictor of high-risk relapse-free survival from statistical analysis (HR: 3.07, 95% CI: 1.07–8.78, $P = 0.036$) [24]. Recently, INT-0116 group reports result of a retrospective analysis on the prognostic value of HER2 in adjuvant therapy choice for gastric cancer. Patients are from INT-0116 phase III gastric cancer clinical trial. Among patients with HER2-nonamplified cancers, treated patients have a median OS of 44 months compared with 24 months in the surgery-only arm ($P = 0.003$). Among patients with HER2-amplified cancers, there is no significant difference in survival based on treatment arm. HER2 status is not a prognostic marker among patients who received no postoperative chemoradiotherapy [25]. In short, molecular biomarkers might be a promising direction to screen the

patients who benefit from postoperative chemoradiotherapy, which warranted further study.

There were several important limitations to our study. First, patient characteristics might be different among selected trials. Cancer stage in the majority of trials was at more advanced stage, including stage II/III, T3/4, and N+ patients, in contrast to the greater proportion of stage I and less proportion of T3/4 patients included in the Nashimoto et al. trial. However, the result of adjusted indirect comparison was not materially altered after omitting this trial (data not shown). Second, because only the published literature and English literature were reviewed for the study, there is the potential for results to be influenced by publication bias and selection bias. Third, the treatment protocols among included RCTs were different with each other. However, due to limited number of the final included RCTs, we did not perform subgroup analysis based on treatment protocols. That is, head to head comparison is needed urgently in future. At last, 6 out of 7 eligible trials were from Asia, making the result less generalized to other region.

5. Conclusions

Based on indirect comparison, chemoradiotherapy demonstrated strong survival advantage over observation in patients with D2-resected gastric cancer. We confirmed the role of adjuvant chemoradiotherapy in D2-resected gastric cancer patients from a different perspective. At present, there are studies which reported that molecular biomarkers might predict clinical outcome to chemoradiotherapy, which was helpful to develop individualized therapy and warranted further study.

Conflict of Interests

There are no financial or nonfinancial competing interests to declare in relation to this paper by any of the authors.

Authors' Contribution

Qiong Yang and Ying Wei contribute equally.

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Research Article

Expression of Potential Cancer Stem Cell Marker ABCG2 is Associated with Malignant Behaviors of Hepatocellular Carcinoma

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Background. Despite improvement in treatment, the prognosis of hepatocellular carcinoma (HCC) remains disastrous. Cancer stem cells (CSCs) may be responsible for cancer malignant behaviors. ATP-binding cassette, subfamily G, member 2 (ABCG2) is widely expressed in both normal and cancer stem cells and may play an important role in cancer malignant behaviors. *Methods*. The expression of ABCG2 in HCC tissues and SMMC-7721 cells was examined, and the relevance of ABCG2 expression with clinical characteristics was analyzed. ABCG2+ and ABCG2− cells were sorted, and the potential of tumorigenicity was determined. Expression level of ABCG2 was manipulated by RNA interference and overexpression. Malignant behaviors including proliferation, drug resistance, migration, and invasion were studied in vitro. *Results*. Expression of ABCG2 was found in a minor group of cells in HCC tissues and cell lines. ABCG2 expression showed tendencies of association with unfavorable prognosis factors. ABCG2 positive cells showed a superior tumorigenicity. Upregulation of ABCG2 enhanced the capacity of proliferation, doxorubicin resistance, migration, and invasion potential, while downregulation of ABCG2 significantly decreased these malignant behaviors. *Conclusion*. Our results indicate that ABCG2 is a potential CSC marker for HCC. Its expression level has a close relationship with tumorigenicity, proliferation, drug resistance, and metastasis ability.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth common cancer in men and the seventh common in women worldwide. Due to its extremely poor prognosis, the deaths and newly diagnosed cases each year are almost equal [1]. Currently, therapeutic strategies for HCC are developing; however, potential curative methods remain surgical resection, transplantation, and radiofrequency ablation [2]. However, according to the widely accepted Barcelona Clinic Liver Cancer (BCLC) staging system, those curative methods are generally limited to early-stage HCC patients, whereas more patients are found with intermediate or advanced stage tumors when diagnosed, thus are not eligible for curative treatment [3]. The effectiveness of noncurative therapies including transcatheter arterial chemoembolization (TACE) and Sorafenib are unsatisfactory, which can only improve overall survival by several months [4]. The dilemma of HCC treatment is largely contributed by the highly malignant behavior of HCC, including early intrahepatic/systemic metastasis and multidrug resistance.

The theory of cancer stem cells (CSCs) is proposed in recent years. According to CSC hypothesis, the formation and progression of cancers are driven by CSCs which represent a minor population in cancer cells [5]. More importantly, CSCs are considered to be responsible for chemotherapy resistance,

metastasis, and postoperative recurrence [6]. Therefore, CSCs may serve as an effective therapeutic target in the treatment of HCC and may improve the current poor prognosis of this disastrous disease. Stem cell phenotypes including self-renewal and the potentiality of differentiation to form heterogeneous cancer cells are deemed to distinguish CSCs from common cancer cells [7]. However, the biomarkers of CSCs are still debatable. Traditional stem cell surface molecules such as CD133, CD44, and CD90 are reported to be markers of CSCs, whereas the results are largely controversial between different studies and tumors from various histological origins [8–10]. On the other hand, side population (SP) cells, which are characterized by efflux of DNA binding dye Hoechst 33342, are also considered as a subpopulation of stem cells in various normal or tumor tissues [11, 12]. Chiba et al. reported that SP cells in HCC xenograft possess extreme tumorigenicity, indicating that this minor population of cells might constitute cancer stem cells in HCC [13]. Further characterization elucidated that ATP-binding cassette (ABC), subfamily G, member 2 (ABCG2), which is widely expressed in various stem cell populations, is highly expressed in SP cells and is responsible for the maintenance of SP phenotype [12]. As an important multidrug resistance transporter, ABCG2 has the capability of efflux various chemotherapy drugs and may contribute to drug resistance of cancer cells [14]. Interestingly, CSCs are also suggested to be responsible for chemoresistance [15]. SP phenotype and chemoresistance strongly imply there is a close association between ABCG2 expression and CSCs maintenance. The conserved expression of ABCG2 in stem cells from both normal tissue and tumor tissues again indicates its important role in stem cell biology [16, 17]. Unfortunately, the effects of ABCG2 expression on CSC-related malignant characteristics are seldom studied.

Our previous studies have reported that the sensitivity to 5-fluorouracil and doxorubicin are negatively correlated with ABCG2 positive rate [18]. In this present study, we investigated the expression of ABCG2 in HCC tissues. Furthermore, we manipulated ABCG2 expression level by RNA interference and plasmid overexpression and studied the effects of ABCG2 expression on HCC malignant behaviors including proliferation, chemoresistance, migration, and invasion.

2. Materials and Methods

2.1. Patients and Specimens. Tumor tissues were obtained from 31 patients with pathologically confirmed HCC at Affiliated Drum Tower Hospital of Nanjing University Medical School. All of the patients received curative resection of HCC between April 2009 and April 2011. Demographic and clinical characteristics such as age, gender, HBV infection status, and alpha fetoprotein (AFP) level, were recorded. Clinical stages of HCC were determined by the TNM staging system of the International Union Against Cancer (Edition 6) [19] and BCLC staging system [3]. Tumor differentiation is graded as poor, moderate, or well. Pathological characteristics including tumor number, tumor size, vascular invasion, and positive rate of Ki67 were determined. We also divided patients by Milan criteria as previously described [20]. This study was approved by the Committee of Ethics of Drum Tower Hospital. Written informed consent was obtained from all of the patients.

2.2. Immunohistochemistry. Formalin-fixed and paraffin-embedded human samples were firstly cut into 5-μm-thick sections. Then the antigen retrieval was accomplished by deparaffinization, rehydration, and boiling in a microwave oven with citrated buffer. 3% hydrogen peroxide in PBS was used to block the endogenous peroxidase activity and BSA was used to block nonspecific staining. Sections were incubated with rabbit anti-ABCG2 polyclonal antibody (1 : 200, 4477S, Cell Signaling Technology, Danvers, MA) and rabbit anti-Ki67 monoclonal antibody (1 : 900, ab16667, Abcam, Cambridge, MA) at 4°C overnight. The EnVision Kit (DAKO, Carpinteria, CA) was used to detect primary antibody followed by staining with DAB reagent and counterstaining with hematoxylin. At last, the slides were photographed with the microscope (BX50, OLYMPUS, Japan). All slides were evaluated by two independent investigators without knowledge of patients' information. Consensus was reached by discussion if different opinion existed. Placenta tissue was used as positive control, and a section with primary antibody omitted served as negative control.

2.3. Cell Culture and Reagents. Human HCC cell line SMMC-7721 was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Gaithersburg, MD) and maintained in 5% CO_2/95%O_2 at 37°C.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and doxorubicin were purchased from Sigma Aldrich (St Louis, MO). β-Actin antibody (4970S) was obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HPR)-conjugated goat anti-rabbit secondary antibody was purchased from MultiSciences Biotech (Hangzhou, China).

2.4. Flow Cytometry. For flow cytometry, cells were detached and incubated with mouse anti-ABCG2 monoclonal antibody (MAB995, IgG2B, R&D Systems, Minneapolis, MN) followed by goat anti-mouse IgG secondary antibody conjugated with FITC (R&D Systems, Minneapolis, MN) for appropriate time. Mouse IgG2B (R&D Systems, Minneapolis, MN) was used as isotype control. A FACSAria flow cytometer (Becton Dickinson, Mountain View, CA) was used to analyze and sort the cells. Cells were sorted by gating FITC-labeled cells compared with isotype control. After sorting, the ABCG2+ and ABCG2− cell fractions were analyzed and purity above 95% was reached.

2.5. Tumorigenic Assay. BALB/c nude mice were maintained in the Animal Experiment Center of Drum Tower Hospital according to the facility's protocol. The protocol of this experiment was reviewed by the local Committee of Ethics. Briefly, mice were randomly divided to receive different number of

ABCG2 positive or negative cells subcutaneously (8×10^3, 4×10^4, 2×10^5, 1×10^6, 5×10^6). After inoculation, tumor formation was observed at 1 week, 2 weeks, 4 weeks, and 8 weeks, respectively. The existence and maximum diameter of tumors were measured and recorded.

2.6. Small Interfering RNA (siRNA) and Plasmid Transfection. siRNA of ABCG2 was synthesized and provided by RIBOBIO (Guangzhou, China). The construction of pcDNA3.1-ABCG2 expression plasmid was described in our previous paper [21]. For transfection, SMMC-7721 cells were plated in 6-well plate and allowed to grow to 70% confluence. Transfection was done using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's guidance.

2.7. Western Blotting. Cell lysates were prepared in RIPA lysis buffer (Beyotime, Nantong, China) with a cocktail of protease inhibitors (Roche, Indianapolis, IN). Total protein concentration was determined by BCA reagent following the manufacturer's instruction (Thermo Scientific, Rockford, IL). 20 μg protein was loaded on each lane of the 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. After gel separation, proteins were transferred to 0.45 μm PVDF membranes (Millipore, Bedford, MA). Thereafter, membranes were incubated with primary antibodies overnight at 4°C following blocking membranes with TBS-T containing 5% nonfat milk. Primary antibodies were removed the next day and the membranes were washed with TBS-T. Membranes were then incubated with HRP-conjugated secondary antibodies for 2 hours at room temperature. After washing the membranes, enhanced chemiluminescence (ECL) reagent (Millipore, Bedford, MA) was applied to the membranes. Specific protein bands were visualized by FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA).

2.8. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was reverse transcribed with a commercial cDNA synthesis kit (Takara Biotechnology, Dalian, China) after extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA templates were amplified with specific primer pairs for ABCG2 and β-Actin using ExTaq polymerase and corresponding buffers (Takara Biotechnology, Dalian, China). The primers used are listed as follows: ABCG2 (forward: 5′-TTATCCGTGGTGTGTCTGGA-3′ and reverse: 5′-TTCCTGAGGCCAATAAGGTG-3′), β-Actin (forward: 5′-GGCATGGGGTCAGAAGGATT-3′ and reverse: 5′-GAGGCGTACAGGGATAGCAC-3′). Agarose gel electrophoresis was carried out with PCR products to analyze the gene expression.

2.9. MTT Assay. The MTT assay was used to determine cell proliferation and doxorubicin sensitivity. Briefly, 5×10^3 cells were planted into 96-well plates. After 24 hours, cells were treated with indicated reagents for different times. 20 μL of MTT (5 mg/mL in PBS) was added and incubated for another 4 hours at 37°C. The MTT formazan precipitate was dissolved in 150 μL of dimethyl sulfoxide after discarding the culture medium. The optical density at 490 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA) to estimate the cell proliferation and doxorubicin sensitivity. When testing doxorubicin sensitivity, IC50 value (defined as concentration of drug when cell viability was inhibited by 50%) was calculated.

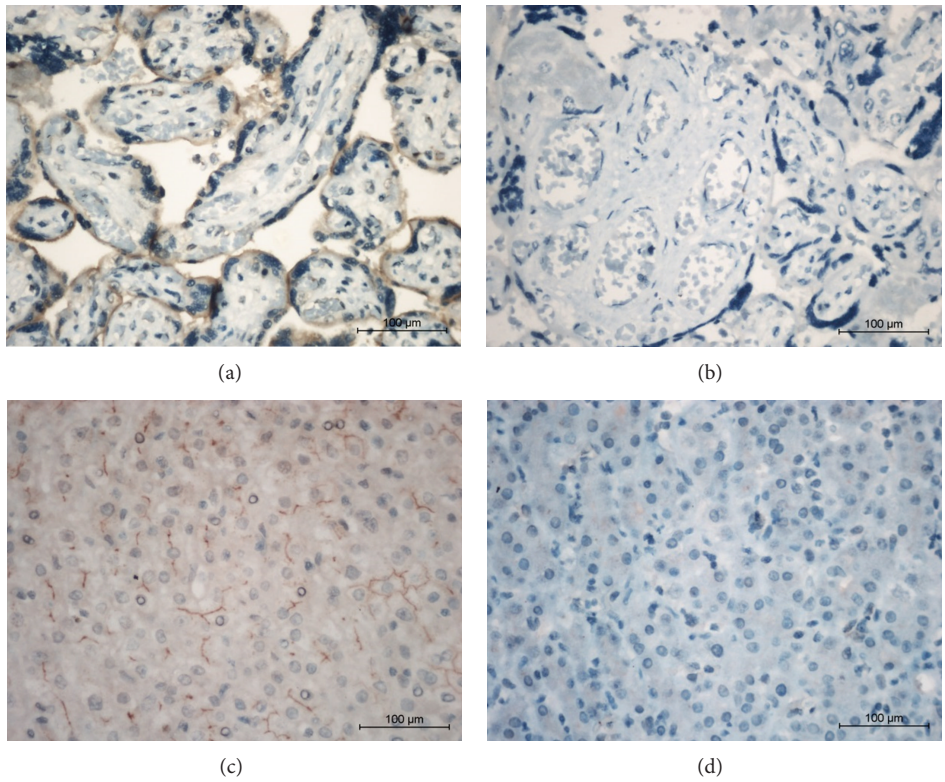
2.10. Wound Healing and Invasion Assay. The wound healing and the transwell invasion assay were performed as previously described [22]. 1×10^5 cells were incubated for 24 hours after seeded in a 12-well plate followed by serum starvation for more than 12 hours. We disrupted the cell monolayers with a 200 μL pipette tip, and took photographs at 0 and 48 hours in a phase contrast microscope. For the transwell invasion assay, Matrigel (Becton Dickinson, Bedford, MA) diluted with serum-free medium were plated to the upper chamber of transwell inserts (Millipore, Bedford, MA). After the Matrigel clotted, 1×10^4 cells were seeded in the upper chamber in serum-free medium, and the lower chamber was filled with the medium containing 10% FBS as a chemoattractant. After 48 hours, the invaded cells were fixed in methanol and the remaining cells in the upper chamber were scratched with a cotton swab. Invaded cells were stained with crystal violet dye and counted under a microscope. Three random visions were counted to calculate the average number.

2.11. Statistical Analysis. Numeric data were expressed as mean ± SD. Difference between two groups was analyzed by two-tailed Student's t-test, and difference among three or more groups was analyzed by one-way analysis of variance multiple comparisons. Categorical data were analyzed by Fisher's exact test. $P < 0.05$ was considered statistically significant.

3. Results

Expression of ABCG2 in HCC Tissue. Immunohistochemistry was done to determine the positive ratio and expression patterns of ABCG2 in 31 HCC tissues. Placenta tissues were used as positive control. As shown in Figure 1, ABCG2 was mainly expressed on the membrane of villous cytotrophoblast. Negative control showed no positive expression and thus confirmed the specificity of ABCG2 antibody. Twenty one out of the 31 HCC samples were detected with expression of ABCG2. The overall positive ratio was 67.74% (Figures 1(c) and 1(d), 21/31). In ABCG2+ HCC tissue, ABCG2 was expressed on cell membrane, which conformed to the expression pattern of this transmembrane protein (Figure 1(c)). It should be noted that only minor cells (approximately 20%) were stained among the cancer cells.

When correlated with patients' demographic and clinical information, ABCG2+ group consisted of more male patients compared with ABCG2− group (95.2% versus 60%, $P = 0.048$). Moreover, ABCG2 expression group showed tendencies towards later BCLC stage, more macrovascular invasion, more patients out of Milan criteria, and higher Ki67 index. However, these tendencies did not reach statistical significance (Table 1).



(a) (b)

(c) (d)

Figure 1: Expression of ABCG2 in human HCC tissues. (a) ABCG2 was expressed in placenta tissues (positive control). (b) Primary antibody omitted placenta tissue slide was set as negative control. (c) Positive ABCG2 expression in HCC tissue. (d) Negative ABCG2 expression in HCC tissue.

3.1. ABCG2 Was Expressed in a Minor Population in SMMC-7721 Cells. As shown in flow cytometry assay, the positive ratio of ABCG2 was 8.8% in SMMC-7721 cells (Figure 2(a)). ABCG2+ and ABCG2− cells were sorted by flow cytometer for subsequent experiments. After 3 passages of culture, the positive ratio of ABCG2 in initial positive cells gradually decreased to 12.7%; however, the positive ratio remained low (1.6%) in negative cells (Figure 2(b)). Western blotting confirmed that ABCG2 expression on protein level was significantly higher (about 10 times) in ABCG2+ cells compared with ABCG2− cells.

3.2. ABCG2+ HCC Cells Displayed High Tumorigenicity In Vivo. In order to evaluate the tumorigenicity of ABCG2+ versus ABCG2− cells, we inoculated subcutaneously 8×10^3, 4×10^4, 2×10^5, 1×10^6, and 5×10^6 ABCG2+ SMMC-7721 cells and the same amount of ABCG2− cells into immunodeficiency mice, respectively. Surprisingly, at 4 weeks after inoculation, all groups of different amounts of ABCG2+ cells formed visible tumors in nude mice while ABCG2− cells failed to establish tumor when inoculated with less than 2×10^5 cells. When only eight thousand cells were grafted into nude mice, ABCG2+ group formed tumors as early as at 2 weeks, and all of the mice developed tumors at 4 weeks after xenograft. However, even inoculated with five times more cells, ABCG2− cells only resulted in one tumor at eight weeks after inoculation (Table 2). Despite the difference of tumorigenicity, tumor sizes were similar between ABCG2+ and ABCG2− groups.

3.3. Silencing of ABCG2 Expression Inhibited the Proliferation and Drug Resistance Potential of HCC Cells. As shown in Figure 4, after sorting, ABCG2+ cells exhibited a higher capacity of proliferation and were more resistant to doxorubicin compared with ABCG2− cells. To explore the impact of ABCG2 on proliferation and drug resistance, we used siRNA method to knockdown ABCG2 expression in ABCG2+ HCC separated by flow cytometer. RT-PCR and western blot were employed to verify the efficiency of RNA interference. Transfection of specific siRNA significantly downregulated the expression of ABCG2 at both mRNA level and protein level (Figures 3(a) and 3(b)). Compared with blank control and the scrambled negative control siRNA, the proliferation was significantly inhibited by siRNA-mediated ABCG2 knockdown (Figure 4(a)). Meanwhile, knockdown of ABCG2 enormously sensitized SMMC-7721 cells to cell deaths induced by doxorubicin (Figure 4(c)). The IC50 value decreased from 1.800 μg/mL to 0.426 μg/mL (Figure 4(e)).

3.4. Upregulation of ABCG2 Led to Elevated Capacity of Proliferation and Drug Resistance. As the silence of ABCG2 expression decreased the proliferation and drug resistance potential, the effect of upregulation of ABCG2 deserved to

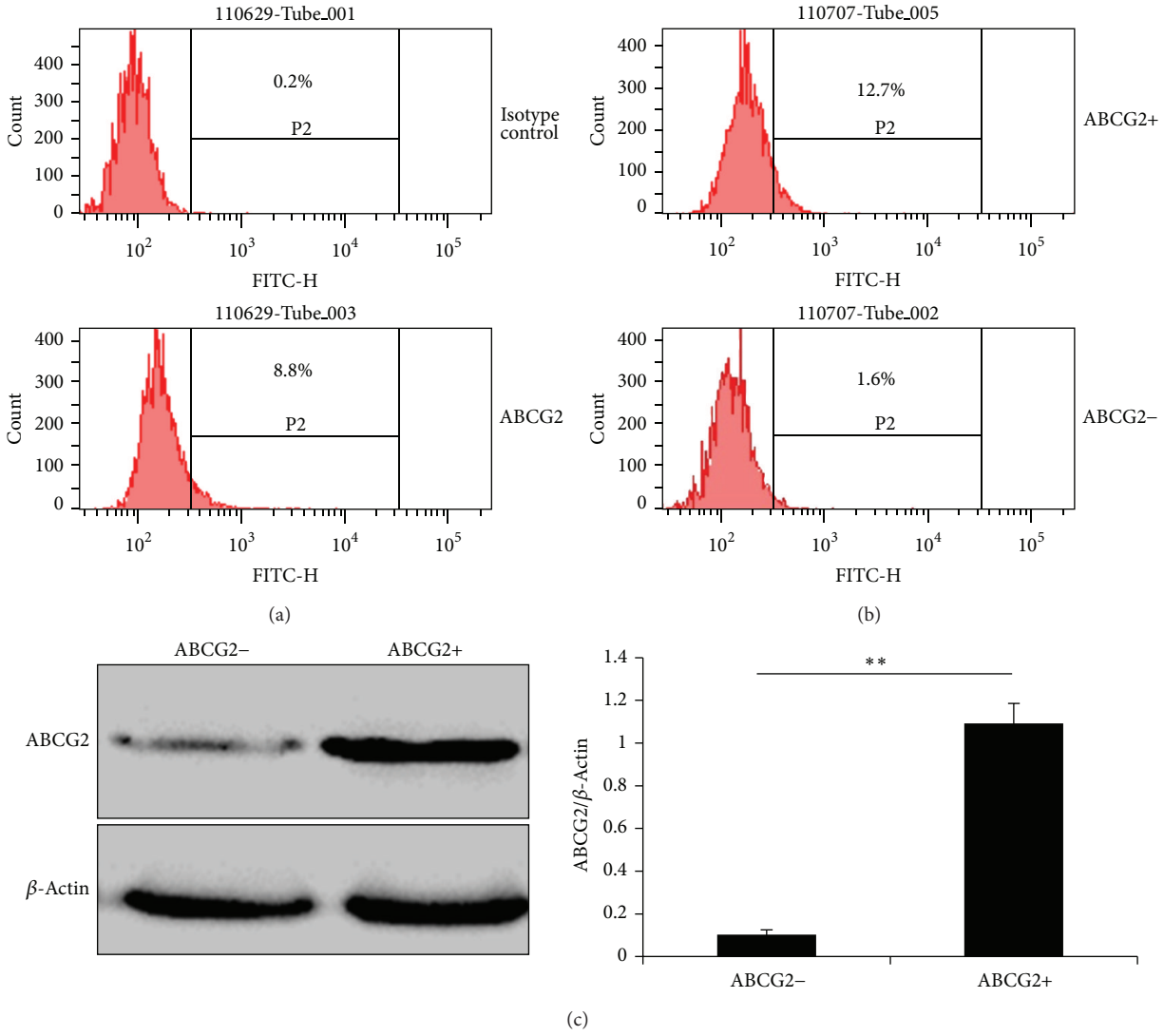


Figure 2: ABCG2 was expressed in a minor population of SMMC-7721 cells. (a) The positive rate of ABCG2 in SMMC-7721 cells determined by flow cytometry was about 8.8% before cell sorting. P2 gate represents ABCG2 positive cells. Mouse IgG2b was used as isotype control. (b) Positive rate of ABCG2 in sorted ABCG2+/ABCG2− cells after 3 passages of culture. (c) Detection of ABCG2 protein level in freshly sorted cells by western blot. β-Actin was used as loading control. The optical density of specific bands was quantified and normalized to β-Actin. $^{**}P < 0.01$.

be studied. We transfected SMMC-7721 cells with ABCG2-expressing plasmid to transiently upregulate the expression of ABCG2 in ABCG2− cells. After transfection, the change of ABCG2 expression was determined by RT-PCR and western blotting. As shown in Figures 3(a) and 3(c), plasmid transfection significantly increased the expression of ABCG2 at both mRNA and protein levels. In contrast to siRNA, overexpression of ABCG2 significantly enhanced the capacity of proliferation in ABCG2 negative SMMC-7721 cells, which reached similar level with ABCG2+ cells (Figure 4(b)). As expected, transient expression of ABCG2 also desensitized ABCG2− cells to doxorubicin (Figure 4(d)). The IC50 level increased from 0.224 μg/mL to 1.888 μg/mL after transfection of ABCG2 (Figure 4(f)).

3.5. Migration and Invasion Ability of ABCG2+ Cells Was Inhibited by RNAi Knockdown. In order to investigate the effect of ABCG2 expression on the ability of migration and invasion, we carried out wound healing migration assay and transwell invasion assay. Freshly after sorting, ABCG2+ cells showed higher ability of migration and invasion than ABCG2− cells. However, after transfected with ABCG2-specific siRNA, the migration of ABCG2+ cells was almost totally inhibited in wound healing assay (Figure 5(a)). Similarly, the invasion potential was also tremendously tempered by the downregulation of ABCG2 (Figure 5(c)).

3.6. Overexpression of ABCG2 Enhanced Migration and Invasion Capacity of ABCG2− Cells. In contrast to ABCG2+

Table 1: ABCG2 expression and characteristics of patients.

	ABCG2 Expression		P-value
	Positive (n = 21)	Negative (n = 10)	
Age			
>50	14 (66.7%)	7 (70%)	0.992
≤50	7 (33.3%)	3 (30%)	
Gender			
Male	20 (95.2%)	6 (60%)	0.027
Female	1 (4.8%)	4 (40%)	
HBsAg			
Positive	14 (66.7%)	7 (70%)	0.992
Negative	7 (33.3%)	3 (30%)	
AFP (ng/mL)			
≤20	8 (38.1%)	2 (20%)	0.420
>20	13 (61.9%)	8 (80%)	
TNM			
I/II	15 (71.4%)	7 (70%)	1.000
III/IV	6 (28.6%)	3 (30%)	
BCLC			
0/A	10 (47.6%)	7 (70%)	0.280
B/C/D	11 (52.4%)	3 (30%)	
Differentiation			
Poor	3 (16.7%)	1 (10%)	1.000
Moderate-Well	18 (83.3%)	9 (90%)	
Tumor number			
Single	17 (81.0%)	8 (80%)	1.000
Multiple	4 (19.0%)	2 (20%)	
Tumor size			
<5	9 (42.9%)	5 (50%)	1.000
≥5	12 (57.1%)	5 (50%)	
Microvascular invasion			
Present	8 (38.1%)	3 (30%)	0.996
Absent	13 (61.9%)	7 (70%)	
Macrovascular invasion			
Present	6 (28.6%)	1 (10%)	0.379
Absent	15 (71.4%)	9 (90%)	
Milan			
In	8 (38.1%)	5 (50%)	0.700
Out	13 (61.9%)	5 (50%)	
Ki67			
≤20%	9 (42.9%)	6 (60%)	0.458
>20%	12 (57.1%)	4 (40%)	

AFP: α-fetoprotein; TNM: TNM staging system of the International Union Against Cancer; BCLC: Barcelona Clinic Liver Cancer staging system; Milan: Milan criteria.

cells, ABCG2− cells showed low migration and invasion ability. However, after the transfection of ABCG2-expressing plasmid, the scratch gap almost completely healed while the wound in control cells remained obvious (Figure 5(b)). Meanwhile, enhancement of the ability of invasion of ABCG2− cell was also observed in transwell invasion assay (Figure 5(d)). This result indicated that expression of ABCG2 enhanced the ability of migration and invasion of HCC cells.

4. Discussion

Despite recent progress in therapeutic strategies, HCC remains incurable to most of patients. Curative resection remains the first choice for the resectable patients [2]. However, recurrences after surgical treatment occur in about 70% of the patients within 5 years [23]. Treatment is limited for patients with recurrent disease and the prognosis is poor.

Table 2: Difference of tumorigenic ability between ABCG2+ and ABCG2− cells.

Cell phenotype	Cell number	Number of tumors established/total number of mice inoculated (maximum diameter of tumors (cm))			
		1 week	2 weeks	4 weeks	8 weeks
ABCG2+	5×10^6	4/4 (0.4–0.6)	4/4 (0.8–1.2)	3/3 (1.6–2.0)	2/2 (1.7–2.1)
	1×10^6	4/4 (0.3–0.5)	4/4 (0.7–1.0)	4/4 (1.2–1.9)	4/4 (0.7–2.0)
	2×10^5	4/4 (0.1–0.3)	4/4 (0.5–0.9)	4/4 (1.0–1.4)	4/4 (1.2–1.5)
	4×10^4	1/4 (0.2)	4/4 (0.5–0.8)	4/4 (0.9–1.3)	3/3 (1.0–2.2)
	8×10^3	0/4	2/4 (0.2–0.5)	4/4 (0.5–1.0)	4/4 (0.6–1.6)
ABCG2−	5×10^6	4/4 (0.2–0.4)	4/4 (0.8–1.1)	4/4 (1.2–1.9)	2/2 (1.3–1.8)
	1×10^6	4/4 (0.2-0.3)	4/4 (0.7–1.0)	4/4 (1.2–1.8)	3/3 (1.3–2.0)
	2×10^5	0/4	3/4 (0.4–0.6)	4/4 (0.6–1.3)	4/4 (0.6–2.5)
	4×10^4	0/4	0/4	0/4	1/4 (0.8)
	8×10^3	0/4	0/4	0/4	0/4

For patients with advanced-stage tumors, only noncurative therapies are available and the effect of treatment is unsatisfactory [24]. Lack of effective therapy for HCC is the main reason for the current dilemma of HCC treatment. However, chemotherapy drug resistance and high metastasis potentiality of HCC limited the efficiency of potential adjuvant therapy.

Cancer stem cells have been extensively studied for the last decade. These cells are thought to be along with invasion, metastasis, and drug resistance potentiality [25]. These characteristics could further result in aggressive phenotype of cancer and poor prognosis. Although the CSCs have been studied for years and therapy against CSCs has been innovated, little assistance has been made to clinical practice. Biomarker uncertainty, complex pathogenesis, and nonspecific expression might lead to this result [26]. Just for HCC, biomarkers such as CD133, CD90, and CD44, are all proposed as CSC markers. Recently, ABCG2 is considered as a potential marker of CSCs in HCC since ABCG2 is the maintaining factor of SP cells which have CSC characteristics and highly detrimental behaviors [27].

Although CSCs only constitute a small portion of tumor, they may have great influence on the biological behaviors of cancer and may determine the prognosis. It is suggested that the expression of CSC-related markers correlate with poor prognosis of HCC [28]. A newly published meta-analysis focused on the relationship between the CSCs and pathological parameters and found their positive relationships [29]. These results indicate that CSCs may play an important role in the malignant behaviors of HCC and may serve as an effective target for the treatment of HCC. ABCG2 was firstly studied as a stem cell marker in bone marrow [12] and its expression was subsequently detected in various cancers [30–33]. Researchers have also revealed its relevance with high tumor stages and poor prognosis [34–36]. However, this molecular target is relatively less studied in HCC. In agreement with the previous study, we found ABCG2 generally expressed in a small population of HCC cells in both tissue and cell lines. This expression pattern in HCC tissues was in accordance with stem/stem-like phenotype as we found the expression of ABCG2 gradually decreased during passage in positive cells. This phenomenon suggested ABCG2+ cells may possess the ability of differentiation as defined by the characteristics of CSC. Our results supported the notion that ABCG2 may be a potential CSC marker in HCC. Furthermore, positive expression in HCC tissues showed tendencies of association with unfavorable clinical and pathological factors including later BCLC stage, more macrovascular invasion, more patients out of Milan criteria, and higher Ki67 index. Although these tendencies were not statistically significant, which may be at least partly due to the limited sample volume in this study, the prognostic role of ABCG2 deserves further study.

According to CSC hypothesis, CSCs are a minor population of cancer cells that possess the ability to form the huge heterogeneous cancer cell pool. It is reported that CSCs have superior ability of tumorigenicity compared with "normal" cancer cells. Chen et al. separated CD133+EpCAM+ cells in one HCC cell line and found their superior tumor formation capacity [37]. Meanwhile, a recent study found that the SP cells sorted from different HCC cell lines identically possessed strong tumorigenicity. These results reveal the correlation between CSCs and tumorigenicity [38]. In agreement with this theory, we found that ABCG2+ cells sorted by flow cytometry presented significantly higher capability of tumorigenicity than negative cells. As less as eight thousand ABCG2+ cells were able to effectively establish visible tumors in nude mice. This observation supported the relationship between ABCG2 expression and malignant behaviors of HCCs in vivo. However, although ABCG2+ cells are more easy to form tumors, the size of tumors was similar to those that arose from ABCG2− cells. This may be because ABCG2+ tumor-initiating cells gradually differentiated and turned into quiescence during the process of tumor growth. We believe that the dynamic change of ABCG2 during different stages of tumor development is worth studying.

Previous studies elucidated that ABCG2 positive HCC cells have higher capacity of proliferation [39]. However, whether the phenomenon has a direct relationship with the expression level of ABCG2 remains unknown. In this study,

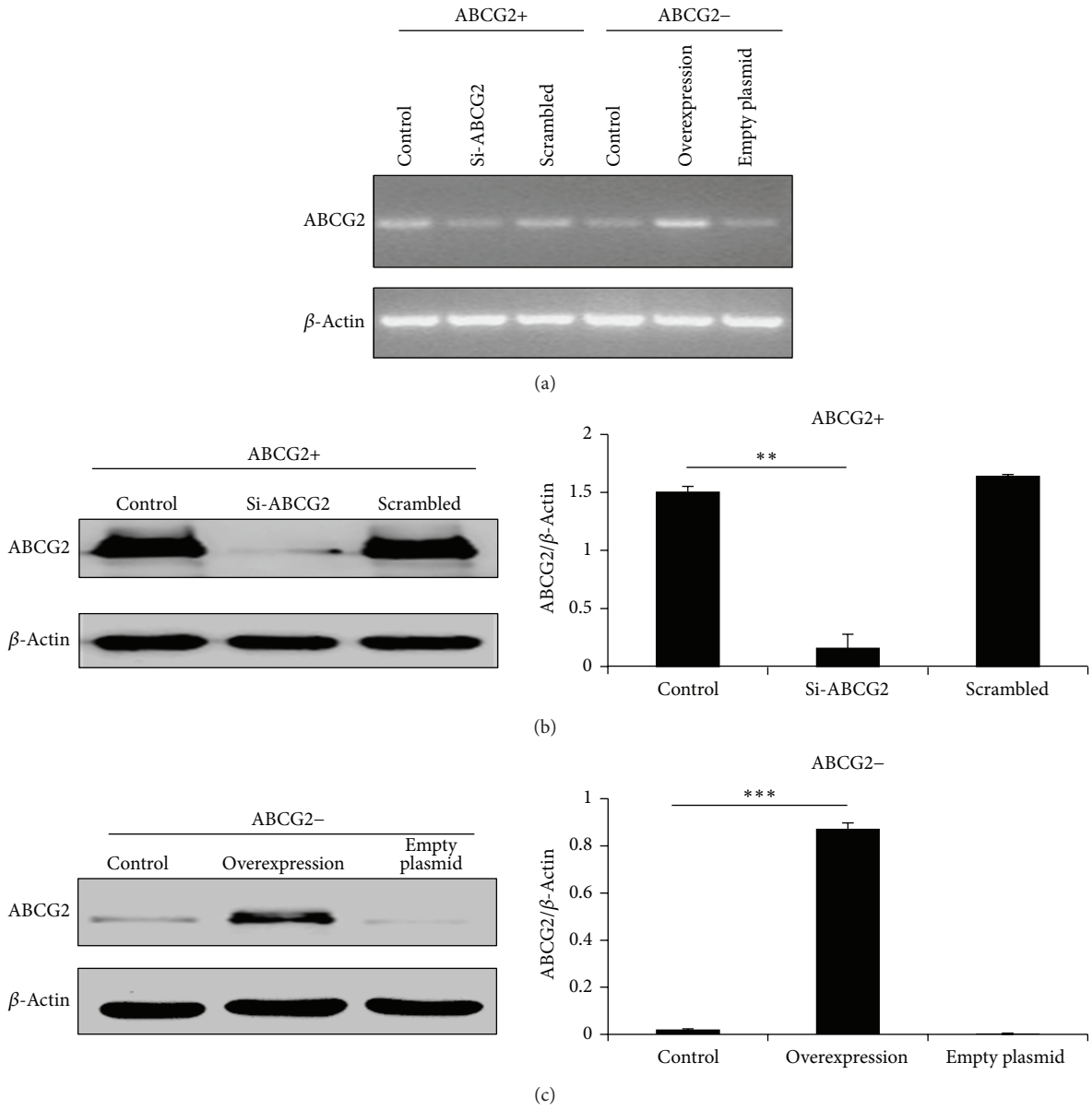


Figure 3: RNA interference and plasmid-mediated overexpression of ABCG2 in SMMC-7721 cells. (a) RT-PCR analysis of ABCG2 mRNA in SMCC-7721 cells after transfection with siRNA or plasmid for 48 h. Normal SMMC-7721 cells were used as blank control. Scrambled siRNA was set as negative control. For overexpression, empty plasmid was transfected as negative control. (b), (c) Western blot analysis confirmed the efficiency of downregulation of ABCG2 by siRNA and upregulation by overexpression, respectively. β-Actin served as loading control. The optical density was quantified and normalized to β-Actin. $^{**}P < 0.01$, $^{***}P < 0.001$.

we also observed that ABCG2+ cells are more proliferative than negative cells. Moreover, we manipulated the expression of ABCG2 by RNA interference and overexpression. Surprisingly, we found that ABCG2 expression had a direct impact on the proliferation of HCC cells. Upregulation of ABCG2 significantly enhanced proliferation while knockdown of this gene tremendously inhibited the growth of highly proliferative ABCG2 positive cancer cells. Our study also showed that ABCG2 expression in tumor tissue correlated with a tendency of a higher Ki67 index, which is a well-established marker of proliferation. The underlying mechanism may include activation of PI3K/Akt and STAT3 signaling pathways [40, 41]. These results indicated that ABCG2 may directly regulate the proliferation of HCC cells. Downregulating or blocking the activity of ABCG2 may represent an effective method for potential treatment of HCC.

The mechanism why CSCs could escape from chemotherapeutics has been investigated. Researchers found that the CSCs often express high levels of ABC drug transporter and these drug efflux pumps might abolish the accumulation of drugs and then reduce the drug efficiency [27]. As one of the most shining stars of the ABC transporter family, ABCG2

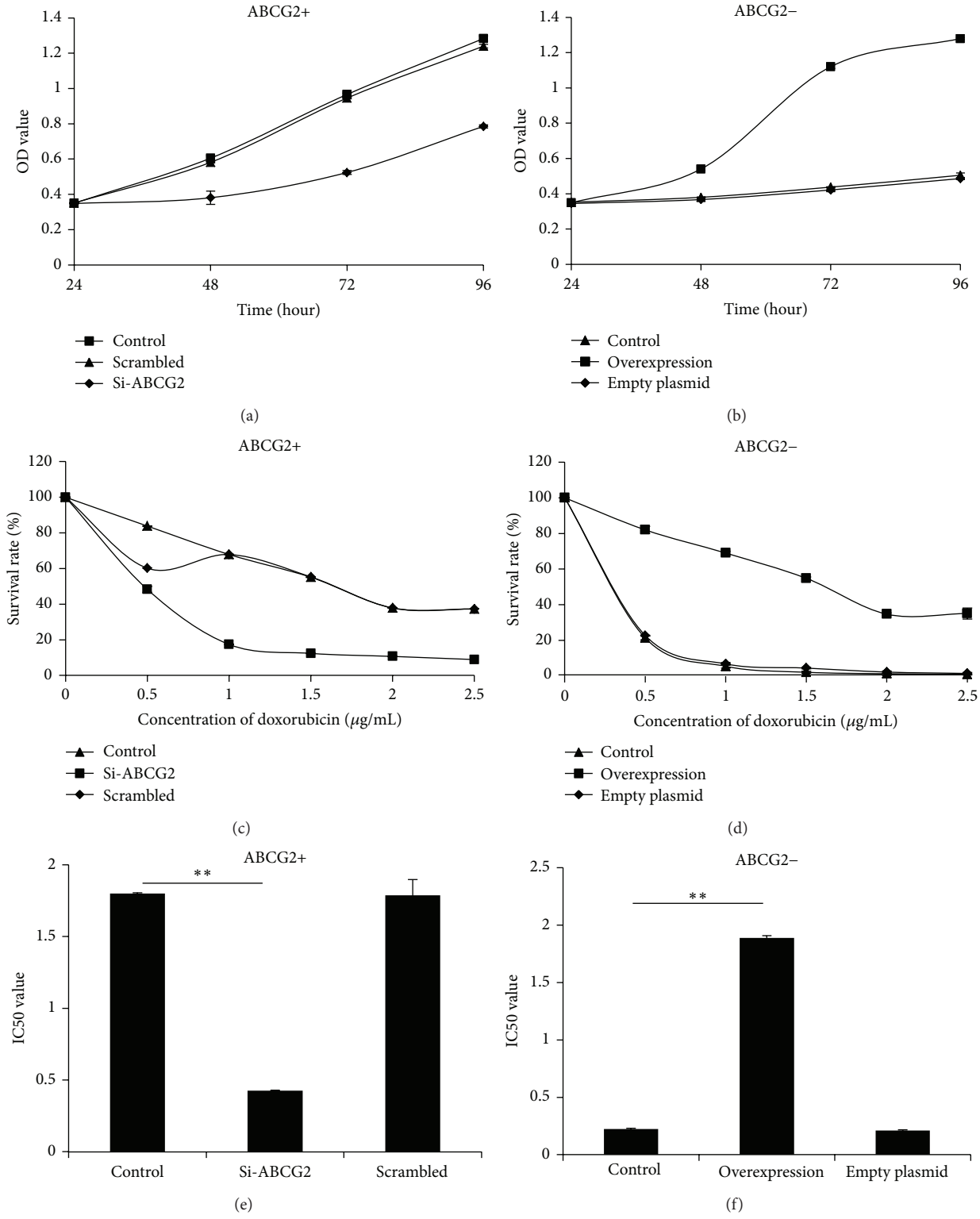


Figure 4: Expression level of ABCG2 correlated with cell proliferation and chemoresistance. (a), (b) Freshly sorted ABCG2+ cells were transfected with ABCG2-specific siRNA. ABCG2− cells were transfected with ABCG2-overexpression plasmid. The proliferation of cells after transfection was compared with untransfected cells and negative control (scrambled siRNA or empty plasmid) using MTT assay after indicated time. (c), (d) The expression level of ABCG2 was manipulated as described above. Survival rates after exposure to indicated dose of doxorubicin for 24 h were determined by MTT assay. (e), (f) IC50 value to doxorubicin was calculated for different groups. $^{**}P < 0.01$.

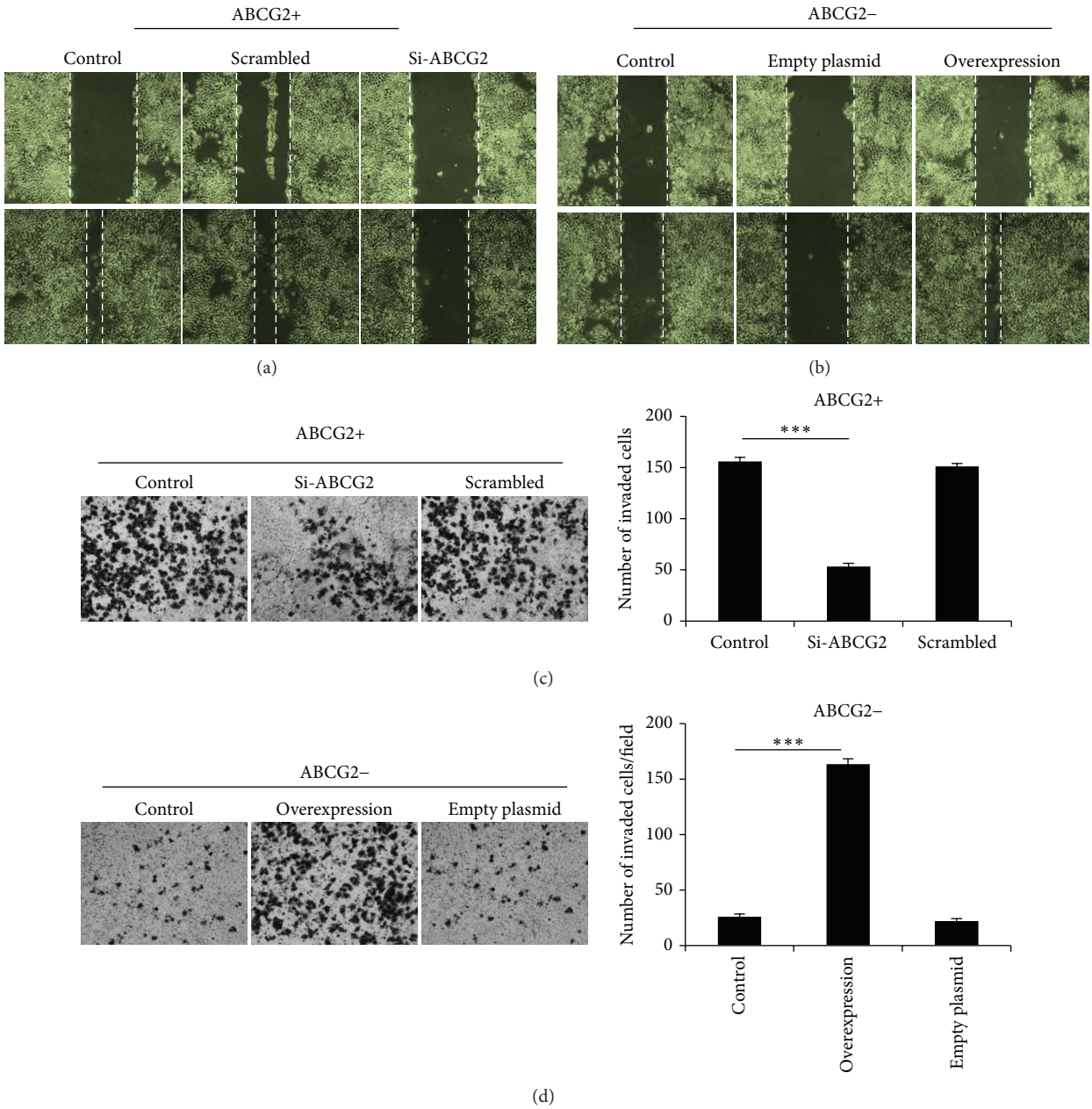


Figure 5: Downregulation or upregulation of ABCG2 had effect on migration and invasion potential in HCC cells. (a), (b) Wound healing assay evaluated the potential of migration of HCC cells. Untreated cells served as normal control. ABCG2+ cells were transfected with ABCG2 siRNA while ABCG2− cells were transfected with ABCG2-expressing plasmid. Scrambled siRNA and empty plasmid were used as negative control. The pictures above represent the wound right after tip scratch. Pictures below showed wound-healing status after 48 h. (c), (d) Results of transwell invasion assay. After 48 h of invasion, cells invaded to the lower chamber were stained and photographed. Invaded cells were counted in three random visions and the average numbers were presented. $^{***}P < 0.001$.

is capable of transporting many substrates including various chemotherapeutics. This characteristic protects tumors from injury of chemotherapy and facilitates drug resistance [27]. In our study, we validated the key role of ABCG2 in chemoresistance. The level of ABCG2 expression correlated with the sensitivity of SMMC-7721 cells to one of the most widely used chemotherapy drugs in HCC-doxorubicin. Manipulation of ABCG2 dramatically changed the effectiveness of this drug on HCC cells. Our results showed that ABCG2 expression level contributed to chemoresistance of HCC. More importantly, ABCG2 may serve as a promising molecular target for the development of adjuvant therapy strategies in combination with traditional chemotherapy.

Among the highly malignant behaviors of HCC, early intrahepatic metastasis and systemic metastasis have largely limited the effect of current treatment of HCC [42]. The

potential of metastasis of cancer cells is determined by the ability of migration and invasion of cancer cells [43]. In HCC, invasive characteristics including incomplete tumor capsules, microvascular and macrovascular invasions, and so forth, are correlated with unfavorable prognosis [4]. Strategies targeting migration and invasion are proposed and may improve the prognosis of cancer by reducing metastasis [44]. CSC phenotype of cancer cells is also correlated with the capability of metastasis. It is now accepted that CSCs may be the source of tumor invasion and metastasis [45]. CSC-related biomarkers are also reported to be associated with invasive behavior of cancer in clinical and pathological studies [46, 47]. In this present study, we found that ABCG2+ cells have significantly higher potential of both migration and invasion in vitro. Inhibition of ABCG2 attenuated these malignant behaviors of ABCG2 positive SMMC-7721 cells. Overexpression of ABCG2 endowed low invasive ABCG2– cells with the ability of migration and invasion. These results correlated the level of ABCG2 expression with the potential of metastasis in HCC cells. Again ABCG2 may be an effective target for the prevention of tumor metastasis and corresponding strategies targeting ABCG2 may help improve the metastatic behavior of HCC.

Our study focused on the role of ABCG2 as a potential CSC marker and its modulatory effect on malignant behaviors of HCC. This present study suggests ABCG2 is expressed in a minor population of HCC cells and ABCG2+ cells manifest some characteristics of CSCs. We also confirm the role of ABCG2 in tumorigenicity, proliferation, drug resistance, migration, and metastasis of HCC. However, further study of ABCG2 in HCC is warranted. Firstly, more pathological specimens should be enrolled to verify the tendencies of association between ABCG2 expression and malignant characteristics of HCC found in this study. Secondly, mechanisms for the changes in biological characteristics should be estimated. At last, particular signaling pathway remains elusive.

In conclusion, ABCG2 participates in malignant behaviors and may serve as a biomarker of CSCs in HCC. It represents a new therapeutic target for solving the current dilemma of HCC treatment.

Author's Contribution

Guang Zhang performed the research, analyzed data, and participated in writing the paper. Zhongxia Wang analyzed data and composed this paper. Weihuan Luo and Hongbo Jiao participated in performing this study. Junhua Wu conceived this study and analyzed this data. Chunping Jiang conceived this study, provided funding, and gave final approval of this paper. Guang Zhang and Zhongxia Wang contributed equally to this study.

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Review Article

Associations of miR-499 and miR-34b/c Polymorphisms with Susceptibility to Hepatocellular Carcinoma: An Evidence-Based Evaluation

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Background. Hepatocellular carcinoma (HCC) represents the sixth common cancer in the world. Single nucleotide polymorphisms (SNPs) in microRNA genes may be associated with susceptibility to HCC. Recently, several studies have reported possible associations of SNPs miR-499 T>C rs3746444 and miR-34b/c T>C rs4938723 with the risk of HCC. However the results are inconsistent and inconclusive. In this present study, we conducted a meta-analysis to comprehensively evaluate potential associations between the two SNPs and HCC susceptibility. *Methods*. Through a systematic literature search, 8-case-control studies involving 5464 subjects were identified and included in this meta-analysis. The association between the two common SNPs and HCC risk was estimated by pooled odds ratios (ORs) and 95% confidence intervals (95% CIs). Our results showed no significant association between rs3746444 and susceptibility to HCC, whereas variant genotypes of rs4938723 were associated with increased HCC risk in allele frequency model and heterozygous model (C versus T, OR = 1.11, 95% CI: 1.01–1.23, $P = 0.04$; TC versus TT, OR = 1.19, 95% CI: 1.03–1.37, $P = 0.02$). *Conclusions*. The current evidence did not support association between rs3746444 and HCC risk. SNP rs4938723 may be associated with susceptibility to HCC. Further well-designed studies are required to clarify the relationships between the two SNPs and HCC risk.

1. Introduction

Hepatocellular carcinoma (HCC) represents the most common primary malignancy of the liver. According to epidemiological survey, the prevalence of HCC ranks the sixth among all cancers. Although the diagnosis and treatment of HCC have significantly been improved in recent years, the prognosis remains poor. HCC accounts for approximately 700,000 cancer-related deaths per year, which ranks the third in global cancer statistics [1, 2]. The mechanism of hepatic carcinogenesis remains elusive. Chronic infection of hepatitis B and hepatitis C viruses (HBV and HCV) and subsequent liver injury-regeneration cycle are considered a major etiology of HCC [3]. However, only a small fraction of chronic viral hepatitis patients finally develop HCC while a considerable portion of HCC cases arise from livers without chronic hepatitis. This fact indicates that the carcinogenesis of HCC is a complex process with multiple factors involved [2, 4].

Recent studies indicate that genetic factors may play important roles in the development of HCC [4]. MicroRNAs (miRNAs) are a group of endogenous small noncoding RNA molecules with length of around 22 nucleotides. It is now clear that miRNAs function as important epigenetic regulators by negatively regulating the stability and transcriptional efficiency of messenger RNAs (mRNAs) [5]. miRNAs are

involved in multiple pivotal processes of cancer development and progression including cell differentiation, proliferation, apoptosis, migration, and invasion [6, 7]. In recent years, the aberrance of miRNAs in cancers, including HCC, has been profiled and many oncogenic or tumor-suppressive miRNAs have been identified [8–10]. Among them, alterations of miRNA-coding genes may be of particular importance. Since one single miRNA regulates a wide spectrum of target genes, even small changes in the amount and function of a miRNA may result in extensive aberrance of tumor-promoter genes and tumor-suppressor genes and finally contribut to carcinogenesis [11].

Single nucleotide polymorphisms (SNPs) are the most common genetic variations in population. Numerous SNPs in or around miRNA-coding genes have been identified, and their roles in human cancer have been implicated [12, 13]. Regulated by p53, both miR-499 and miR-34b/c are recognized as important factors in the process of carcinogenesis, apoptosis, and metastasis [14–16]. Recently, several molecular epidemiological studies have investigated potential associations of common SNPs rs3746444 in miR-499 [17–21] and rs4938723 in the promoter region of pri-miR-34b/c gene [22–24] with risk of HCC. Unfortunately, the results in these studies are controversial and inconclusive. Since individual study may be underpowered and biased for effective evaluation of potentially slight effects of the two functional polymorphisms due to relatively limited study population, a meta-analysis was conducted to achieve more comprehensive estimation of associations between the two common SNPs and susceptibility to HCC with up-to-date evidence.

2. Methods

2.1. Literature Search Strategy. A comprehensive computerized literature search was conducted in multiple online databases including PubMed, EMBASE, ISI Web of Science, the Cochrane Library, China National Knowledge Infrastructure (CNKI), Wanfang Database, and VIP Info database with the combination of the following search terms: "miR-499," "miR-34," "rs3746444," "rs4938723," "hepatocellular carcinoma," "liver cancer," "hepatoma," and "HCC" to identify potentially relevant studies. The literature search was performed by two independent investigators (Zhongxia Wang and Junhua Wu, last update: July 15, 2013). Publication date, language, full article, or abstract was not restricted in our search. The reference lists of eligible studies were manually searched as a complement to electronic search. The results of search were crosschecked, and final consensus was reached between the two investigators.

2.2. Study Selection. Studies in our meta-analysis had to meet all of the following criteria: (1) case-control design; (2) evaluating the association between SNPs miR-499 T>C (rs3746444) and/or miR-34b/c T>C (rs4938723) and susceptibility to HCC; (3) being studied on human beings; (4) sufficient data of allele and genotype frequencies of SNPs being provided for the estimation of odds ratios (ORs) and 95% confidence intervals (95% CIs); (5) only the most recent study being included if serial studies of the same population were reported; and (6) methodological design being proper. Methodology of potential relevant case-control studies was evaluated using the following criteria: (1) the demographic data were comparable between case and control groups; (2) the diagnosis of HCC was determined with proper diagnostic methods; (3) appropriate methods were employed to determine genotypes; and (4) proper statistical methods were used. The details of study search and selection were provided in Figure 1 by flow diagram.

2.3. Data Abstraction. Data were extracted from eligible studies by two independent investigators (Zhongxia Wang and Junhua Wu). The extracted data included the authors' name, publication date, origin of the study, ethnicity of studied population, methods of genotyping, allele, and genotype frequencies in both case and control groups. The two investigators crosschecked the results of data abstraction. When different results were obtained, they repeated data extraction of the specific study and discussed them to reach mutual agreement. If disagreement still existed, two senior investigators (Yitao Ding and Chunping Jiang) were invited to discussion until consensus was reached.

2.4. Data Synthesis and Statistical Analysis. Chi-square test was used to test whether the distribution of genotypes in control group deviated from HWE. ORs and corresponding 95% CIs were calculated to evaluate the strength of associations between SNPs and the risk of HCC under the following genetic models: (1) allele frequency (C versus T); (2) heterozygous model (TC versus TT); (3) homozygous model (CC versus TT); (4) dominant model (TC + CC versus TT); and (5) recessive model (CC versus TT + TC). Heterogeneity among included studies was evaluated by Cochrane Q-test and $P > 0.10$ indicated that no significant statistical heterogeneity existed [25]. Pooled ORs and 95% CIs were generated to estimate the effect of rs3746444 and rs4938723 on susceptibility to HCC. If no significant heterogeneity was detected, the pooled ORs were generated by fixed-effects model (Mantel-Haenszel method) [26]. Otherwise, the random-effects model (DerSimonian-Laird method) was used to estimate pooled statistics [27]. The significance of pooled ORs was determined by Z-test. A P value less than 0.05 was considered as statistically significant. Corresponding forest plots were generated to show the results of meta-analyses.

Sensitivity analysis was conducted by excluding individual study in turn to observe the change in heterogeneity test and the significance of pooled ORs. For miR-499 T>C (rs3746444), subgroup analysis was carried out using data from Asian ethnicity, HBV-infected cases and studies within HWE. All of the data synthesis and statistical analysis were performed using Review Manager 5.2.5 software (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012).

3. Results

3.1. Study Characteristics. As shown in Figure 1, 121 potentially relevant articles were retrieved by computerized

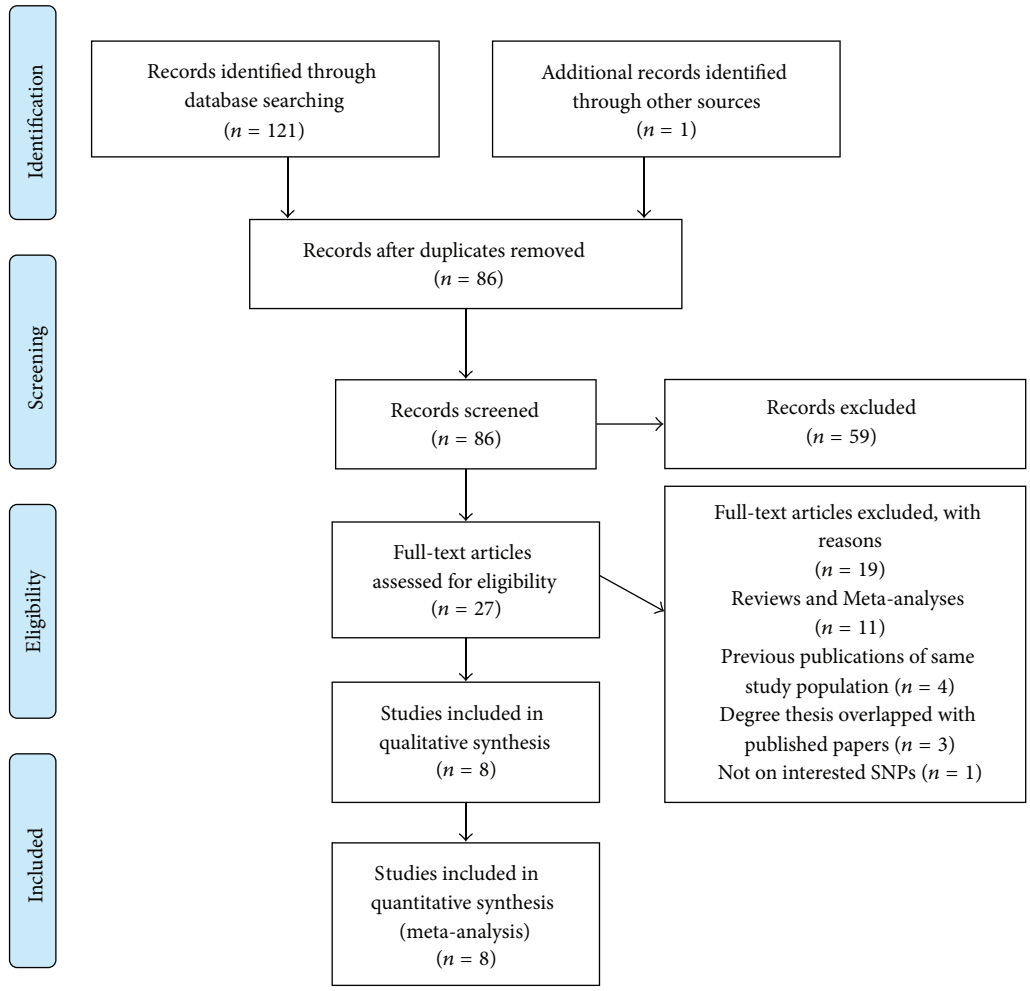


Figure 1: Flow diagram of literature search and selection.

database search. Reference list examination further identified another relevant study. After the removal of duplicates, 86 records were screened by reviewing titles and abstracts. According to inclusion criteria, 59 records were excluded. The remaining 27 full-text articles were retrieved and assessed. Eight eligible studies were finally included in this meta-analysis [17–24].

Characteristics of included studies are shown in Table 1. Five studies on miR-499 T>C rs3746444 involving 852 cases and 1191 controls were included in this meta-analysis [17–21]. For miR-34b/c T>C rs4938723, a total of three studies reported potential association between this SNP and the risk of HCC with evidence from 1672 cases and 1749 controls [22–24]. All of the studies except the report from Akkiz et al. [17] were carried out in Asian population. The method of genotyping included polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real-time polymerase chain reaction. For the studies from Akkiz et al. [17] and Zou and Zhao [21], genotype distribution in control group deviated from HWE. Genetic distributions in control groups of the rest studies conformed to HWE.

3.2. Lack of Association between miR-499 T>C rs3746444 and Susceptibility to HCC. The association between rs3746444 and the risk of HCC was analyzed using data from five independent studies [17–21]. The results of this meta-analysis were summarized in Table 2. Significant statistical heterogeneity was detected in all of the genetic models except heterozygous model. Therefore the random-effect model was employed to calculate the pooled ORs. To our surprise, the results demonstrated no significant association between rs3746444 and the risk of HCC in any genetic model tested. Sensitivity analysis revealed that the study from Xiang et al. [19] was the main source of statistical heterogeneity since the significance of Q-test became negative after the exclusion of this study. However, there was still no obvious association between rs3746444 and susceptibility to HCC in the remaining studies with less heterogeneity.

Subgroup analyses were conducted in Asians, HBV infected HCC patients and in studies that conformed to HWE. Similarly, miR-499 T>C rs3746444 was not associated with HCC susceptibility in all genetic model analyzed in Asians. Four studies provided genotypes in HBV-associated

Table 1: Characteristics of included studies.

Author	Year	Country	Ethnicity	SNP	Genotyping methods	*P* for HWE	Case genotype			Control genotype		
							TT	TC	CC	TT	TC	CC
Akkiz	2011	Turkey	Caucasian	rs3746444	PCR-RFLP	0.036	45	87	90	47	93	82
Kim	2012	Korea	Asian	rs3746444	PCR-RFLP	0.278	109	47	3	120	74	7
Xiang	2012	China	Asian	rs3746444	PCR-RFLP	0.284	36	40	24	54	36	10
Zhou	2012	China	Asian	rs3746444	PCR-RFLP	0.620	141	41	4	371	100	12
Zou	2013	China	Asian	rs3746444	PCR-RFLP	0.005	136	44	5	123	48	14
Xu	2011	China	Asian	rs4938723	Real-time PCR	0.647	204	236	62	266	229	54
Son	2013	Korea	Asian	rs4938723	PCR-RFLP	0.371	69	75	13	110	74	17
Han	2013	China	Asian	rs4938723	PCR-RFLP	0.183	451	444	118	456	424	119

SNP: single nucleotide polymorphism; *P* for HWE: *P* value for Hardy-Weinberg equilibrium (HWE) calculated by Chi-square test. $P < 0.05$ indicates deviation of genotype distribution from HWE.

Table 2: Meta-analysis of the association between SNP miR-499 (T>C) rs3746444 and susceptibility to HCC.

Genetic model	Population	OR	95% CI	*Z*	*P*	*P*-h
Allele frequency C versus T	Overall	1.01	0.72–1.43	0.06	0.95	0.0008
	Asian	0.99	0.62–1.59	0.04	0.97	0.0004
	HBV infected	0.96	0.59–1.56	0.16	0.87	0.001
	HWE	1.13	0.64–2.01	0.43	0.67	0.001
Heterozygous model TC versus TT	Overall	0.96	0.77–1.19	0.39	0.70	0.23
	Asian	0.95	0.75–1.21	0.38	0.70	0.13
	HBV infected	0.83	0.54–1.28	0.83	0.40	0.09
	HWE	1.04	0.66–1.63	0.17	0.86	0.07
Homozygous model CC versus TT	Overall	0.96	0.44–2.09	0.10	0.92	0.006
	Asian	0.87	0.27–2.85	0.23	0.82	0.003
	HBV infected	1.04	0.45–2.43	0.10	0.92	0.04
	HWE	1.24	0.36–4.34	0.34	0.73	0.02
Dominant model TC + CC versus TT	Overall	1.00	0.71–1.42	0.00	1.00	0.02
	Asian	0.99	0.63–1.55	0.03	0.97	0.009
	HBV infected	0.90	0.53–1.53	0.40	0.69	0.01
	HWE	1.12	0.63–1.99	0.37	0.71	0.009
Recessive model CC versus TT + TC	Overall	0.97	0.50–1.88	0.08	0.93	0.02
	Asian	0.86	0.30–2.45	0.28	0.78	0.009
	HBV infected	1.23	0.86–1.75	1.15	0.25	0.12
	HWE	1.22	0.43–3.47	0.37	0.71	0.06

OR: odds ratio; 95% CI: 95% confidence interval; *Z*: *Z* value for *Z*-test; *P*: *P* value for *Z*-test; *P*-h: *P* value for *Q*-test; HBV infected: subgroup analysis in hepatitis B virus (HBV) infected cases. HWE: only studies that conform to Hardy-Weinberg equilibrium were included in this subgroup analysis.

HCC patients [17–19, 21]. Subgroup analysis showed that SNP rs3746444 did not modify the risk of HCC in the patients with chronic HBV infection. Likewise, for studies within HWE, which were of less selection bias, consistent negative results were confirmed.

3.3. Association between miR-34b/c T>C rs4938723 and Susceptibility to HCC. Three studies reported potential association between rs4938723 and HCC risk with evidence from 1672 cases and 1749 controls [22–24]. All of these studies were carried out in Asian population. Results of this meta-analysis were shown in Table 3. Fixed-effect model was used in most genetic models to estimate pooled ORs and 95% CIs since no significant heterogeneity was detected except for dominant model. Significant associations of rs4938723 with the risk of HCC were observed in allele frequency model and heterozygous model (Figure 2). Compared with T allele, C variant of rs4938723 was associated with a higher risk of HCC (OR = 1.11, 95% CI: 1.01–1.23, $P = 0.04$). In heterozygous model, carriers of TC genotype were more susceptible to HCC compared with TT carriers (OR = 1.19, 95% CI: 1.03–1.37, $P = 0.02$). A trend of association was also observed in dominant model although it did not reach statistical significance with a marginal *P* value of 0.06 (OR = 1.25, 95% CI: 0.99–1.58). No significant association was demonstrated in homozygous model and recessive model.

Table 3: Meta-analysis of the association between SNP miR-34b/c (T>C) rs4938723 and susceptibility to HCC.

Genetic model	OR	95% CI	*Z*	*P*	*P*-h
Allele frequency C versus T	1.11	1.01–1.23	2.10	0.04	0.11
Heterozygous model TC versus TT	1.19	1.03–1.37	2.40	0.02	0.12
Homozygous model CC versus TT	1.15	0.92–1.44	1.22	0.22	0.28
Dominant model TC + CC versus TT	1.25	0.99–1.58	1.85	0.06	0.09
Recessive model CC versus TT + TC	1.06	0.86–1.31	0.55	0.58	0.49

OR: odds ratio; 95% CI: 95% confidence interval; *Z*: *Z* value for *Z*-test; *P*: *P* value for *Z*-test; *P*-h: *P* value for *Q*-test.

4. Discussion

Despite significant advancements in the research of HCC, the detailed etiology of this fatal disease remains elusive. Besides well-known risk factors, such as viral hepatitis, alcohol abuse, and nonalcoholic fatty liver disease (NAFLD), genetic factors may also contribute to the development of HCC [4, 28]. Identification of genetic biomarkers of HCC susceptibility may be extremely valuable in facilitating early diagnosis and discovering molecular targets for personalized treatment. As important epigenetic regulators, miRNAs are crucial in the process of liver carcinogenesis by acting as either oncogenes or tumor-suppressor genes [29]. SNPs represent the most common genetic polymorphisms in human genome. Through altering the expression, stability, and function of miRNAs, SNPs may indirectly affect a wide range of cancer-related genes and thus play important role in individual's susceptibility to HCC [30, 31]. Therefore, SNPs of miRNA-coding genes may serve as genetic biomarkers of HCC risk. miR-499 and miR-34b/c are regulated by tumor-suppressor gene p53 and may participate in hepatic carcinogenesis [15, 16]. Recently, two functional SNPs miR-499 T>C rs3746444 and miR-34b/c T>C rs4938723 have been reported to associate with HCC susceptibility [17–24], indicating potential value of these SNPs in risk screening of HCC. However results from these studies are controversial and inconclusive, likely because of the limited sample size of individual study and potential selection bias. In this present meta-analysis, we systematically analyzed eight independent case-control studies and reevaluated the potential association of the two common SNPs with susceptibility to HCC.

miR-499 participates in several crucial cancer-related biology processes such as apoptosis, cell migration, cell senescence, and inflammation [32–34]. Regulated by p53, miR-499 inhibits apoptosis by targeting calcineurin and dynamin-related protein-1 (Drp1). More importantly, Lafferty-Whyte et al. revealed that miR-499 functions as a prometastatic miRNA. It promotes the migration and invasion of colorectal cancer cells via regulating the expression of forkhead box O4 (FOXO4) and programmed cell death 4 (PDCD4) [34]. Together with miR-34c, this miRNA also regulates multiple types of cellular senescence induction [32]. miR-499 T>C polymorphism (rs3746444) results in an alteration from A:U pair to G:U mismatch in the stem region of miR-499, which reduces secondary structure stability and affects the process of miRNA maturation as well as binding to target mRNA [35]. Therefore, rs3746444 may contribute to susceptibility to cancer by regulating downstream genes. Positive associations between rs3746444 and the risks of various cancers have been reported [35–37]. Five studies demonstrated inconsistent results of association between this SNP and HCC risk. Three of them suggested significant association [18, 19, 21] while the rest studies reported no obvious association observed [17, 20].

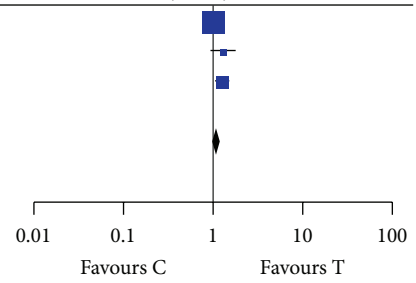
In this meta-analysis, we assessed possible association between SNP rs3746444 and susceptibility to HCC with evidence from 852 cases and 1191 controls. Our results elucidated that there lacks any association between rs3746444 and the risk of HCC in all of genetic models tested. Sensitivity analysis revealed that the study from Xiang et al. [19] was the main source of heterogeneity. However, the association remained negative even this study was excluded. To address effects of potential confounding factors on the results of this meta-analysis, we performed several subgroup analyses. We firstly conducted subgroup analysis in Asian population since the incidence of SNP may vary between different ethnicity. After that, studies conformed to HWE were synthesized to rule out selection bias. Stratified analysis by HBV infection status was also carried out to identify potential interaction between SNP and HBV infection. Consistently, no significant association was observed in all of the subgroup analyses. The results of this meta-analysis do not support a genetic association of rs3746444 with susceptibility to HCC. Sensitivity analysis and subgroup analyses confirmed the stability of the results.

As members of the miR-34 family, miR-34b and miR-34c share a common primary transcript (pri-miR-34b/c). Induced by p53 in response to genotoxic stress, miR-34b/c are considered as tumor-suppressor miRNAs [15, 38]. Previous studies revealed that the miR-34 family acts as negative regulators of hundreds of oncogenes and subsequently induce cell cycle arrest or cell death [15, 39]. Gene therapy using miR-34 as target molecule was proven effective for HCC in animal models [38]. In patients, downregulation of miR-34b/c by CpG islands methylation in the promoter region was frequently observed in cancers from various histological sources [40–45]. SNP rs4938723 (T>C) is located within CpG islands in the promoter of pri-miR-34b/c and may participate in epigenetic silence of miR-34b/c. Furthermore, this T>C variant is also predicted to affect the binding of GATA-X transcription factors to the promoter region of pri-miR-34b/c gene [22]. Hence rs4938723 may alter the promoter transcription activity, affect the expression level of miR-34b/c, and subsequently cause change in susceptibility to cancer [18]. Indeed, this SNP is reported to be associated with susceptibility to colorectal cancer [46], nasopharyngeal carcinoma [47], breast cancer [48], and HCC [22–24]. Although all studies on HCC demonstrated association between rs4938723 and HCC risk, the genetic models with statistical significance were inconsistent.

In the present study, we systematically evaluated the potential association of rs4938723 with susceptibility to HCC with data from 1672 cases and 1749 controls. Statistically

Study or subgroup	C Events	C Total	T Events	T Total	Weight	Odds ratio M-H, fixed, 95% CI
Han (2013)	680	1342	1346	2682	62.1%	1.02 [0.89, 1.16]
Son (2013)	101	209	213	507	9.0%	1.29 [0.93, 1.78]
Xu (2011)	360	697	644	1405	28.9%	1.26 [1.05, 1.51]
Total (95% CI)		**2248**		**4594**	**100.0%**	**1.11 [1.01, 1.23]**
Total events	1141		2203			

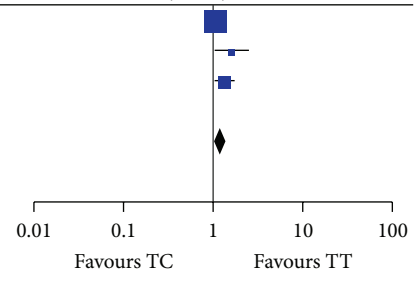
Heterogeneity: $\chi^2 = 4.37$, df = 2 ($P = 0.11$); $I^2 = 54\%$
Test for overall effect: $Z = 2.10$ ($P = 0.04$)



(a)

Study or subgroup	TC Events	TC Total	TT Events	TT Total	Weight	Odds ratio M-H, fixed, 95% CI
Han (2013)	444	868	451	907	62.2%	1.06 [0.88, 1.28]
Son (2013)	75	149	69	179	9.0%	1.62 [1.04, 2.51]
Xu (2011)	236	465	204	470	28.8%	1.34 [1.04, 1.74]
Total (95% CI)		**1482**		**1556**	**100.0%**	**1.19 [1.03, 1.37]**
Total events	755		724			

Heterogeneity: $\chi^2 = 4.22$, df = 2 ($P = 0.12$); $I^2 = 53\%$
Test for overall effect: $Z = 2.40$ ($P = 0.02$)



(b)

Figure 2: Forest plots of meta-analysis of association between rs4938723 and the risk of HCC. (a) Meta-analysis under allele frequency model. (b) Meta-analysis under heterozygous model. The blue squares and corresponding horizontal lines indicate odds ratio of individual study. The area of the squares reflects weight of indicated study. The black filled diamond represents pooled odds ratio and 95% confidence interval.

significant associations were demonstrated in analyses under allele frequency (C versus T) and heterozygous model (TC versus TT). Genetic variant T>C increases the risk of HCC in these two genetic models. A trend of elevated risk of HCC was observed in dominant model (TC + CC versus TT) although this trend did not reach statistical significance with a marginal P value of 0.06. Importantly, obvious heterogeneity existed in the estimation of dominant model. However, limited number of eligible studies and lack of detailed information prevented us from exploring the source heterogeneity by subgroup analysis. Possible association between rs4938723 and the risk of HCC under dominant model should not be rule out.

To the best of our knowledge, this is the first meta-analysis evaluating the association between miR-34b/c T>C rs4938723 and susceptibility to HCC. This is also the latest study assessing potential association between miR-499 T>C rs3746444 and HCC risk with the most evidence, though the conclusion was in agreement with previous meta-analyses [49–51]. However, the results of this study should be interpreted cautiously due to several limitations. Firstly, even after synthesizing all available data, the sample size remains relatively small. This may limit the power of this study to detect potential slight effects of these two SNPs on HCC susceptibility. The limited number of included studies also prevented us from evaluating publication bias by funnel plot. It should be noted that potential publication bias may introduce confounders and the conclusion might deviate from true effect. Moreover, heterogeneity was found significant in most analyses of rs3746444. Although sensitivity analysis and subgroup analyses confirmed the stability of our results, it should be acknowledged that inherent heterogeneity may distort the true effect and may reduce the reliability of the results. Finally, there was no sufficient data regarding age, gender, smoking, alcohol consumption, the status of fatty liver diseases, and so forth, for clarifying their effects on HCC susceptibility and interactions with SNPs. These factors may confound with true effects of SNPs and conceal or exaggerate the possible associations.

In conclusion, the SNP rs4938723 in the promoter region of pri-miR-34b/c gene is associated with elevated susceptibility to HCC. However, our meta-analysis does not support association between rs3746444 and the risk of HCC. Further well-designed studies with larger sample size are required to verify our findings. Functional studies and interactions of these two SNPs with other predisposing or protective factors of HCC will be of great interest. The value of SNP rs4938723 as a genetic biomarker for early diagnosis or a molecular target for novel therapy of HCC is also worthy of investigating.

Author Contributions

Yitao Ding and Chunping Jiang conceived this work and provided funds. Zhongxia Wang, Junhua Wu, Guang Zhang, and Yin Cao performed the research and analyzed the data.

Zhongxia Wang, Junhua Wu, and Chunping Jiang composed this paper. Zhongxia Wang and Junhua Wu contributed equally to this paper.

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Clinical Study

MicroRNA-429 Modulates Hepatocellular Carcinoma Prognosis and Tumorigenesis

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MicroRNA-429 (miR-429) may modify the development and progression of cancers; however, the role of this microRNA in the hepatocellular carcinoma (HCC) has not been well elaborated. Here, we tested miR-429 expression in 138 pathology-diagnosed HCC cases and SMMC-7721 cells. We found that miR-429 was upregulated in HCC tumor tissues and that the high expression of miR-429 was significantly correlated with larger tumor size (odd ratio (OR), 2.70; 95% confidence interval (CI), 1.28–5.56) and higher aflatoxin B1-DNA adducts (OR = 3.13, 95% CI = 1.47–6.67). Furthermore, this microRNA overexpression modified the recurrence-free survival and overall survival of HCC patients. Functionally, miR-429 overexpression progressed tumor cells proliferation and inhibited cell apoptosis. These results indicate for the first time that miR-429 may modify HCC prognosis and tumorigenesis and may be a potential tumor therapeutic target.

1. Introduction

Primary liver cancer is the sixth most commonly occurring cancer worldwide, with an estimated 600,000 new cases per year [1–3]. Because of the very poor prognosis and the same number of deaths, this tumor is the third most common cause of cancer deaths in the world [2, 3]. Liver cancer is histopathologically classified into two major types, hepatocellular carcinoma (HCC) and cholangiocellular carcinoma. HCC often exhibits blood metastasis and recurrence [4–6]. Therefore, improvement in the therapy of recurrent or metastatic HCC now depends on improving our understanding of the complex molecular mechanisms governing the progression and aggressiveness of the disease.

Over the last several decades, it has been recognized that multiple risks, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, chemical carcinogen aflatoxin B1 (AFB1), and genetic abnormalities, are implicated in the multistep process of liver carcinogenesis [5–7]. Increasing evidence has shown that microRNA may play an important role in the tumorigenesis of this malignant [8–12], while microRNA-429 (miR-429), a member of the microRNA-200 family of microRNAs, can hinder the expression of transcriptional repressors SIP1/ZEB213 and ZEB1/deltaEF1 and regulate epithelial-mesenchymal transition [13, 14]. Recent studies have shown that downregulation of miR-429 may be an important late step in tumour progression [14]. Increasing data exhibit that dysregulation of this microRNA expression can modify tumor prognosis possibly through regulating cell proliferation and apoptosis [13, 15–18]. However, association between this microRNA and HCC has not yet been elucidated. Here, we evaluated whether miR-429 expression modified HCC clinicopathological features and prognosis and explored the effects of this microRNA on cancer cell proliferation and apoptosis.

2. Materials and Methods

2.1. Patient and Followup. This study was approved by the ethics committees of the hospitals involved in this study. All activities involving human subjects were carried out under full compliance with government policies and the Helsinki Declaration. A total of 138 HCC patients, including 65 patients previously studied [19, 20], were included in the present study. All cases were identified through hepatosurgery, hepatopathology, oncology, hepatology centers, and through cancer registries in the affiliated hospitals of the two main medical colleges in Guangxi (namely, Guangxi Medical University and Youjiang Medical College for Nationalities) between January 2004 and December 2005. All of the cases were confirmed by histopathological diagnosis in 100% of the HCC cases with the I-II tumor-nodes-metastasis (TNM) stage and had undergone the same curative resection treatment, according to Chinese Manage Criteria of HCC [21]. After giving written consent, demographic information (including sex, age, ethnicity, and HBV and HCV infection) and clinical pathological data (including cirrhosis, tumor size, and tumor stage) were collected in the hospitals using a standard interviewer-administered questionnaire and/or medical records [19, 20, 22]. Surgically removed tumor samples and adjacent noncancerous tissue samples (at least 5 cm from the margin of the tumor) of all cases were collected for analyzing miR-429 expression levels and AFB1-DNA adduct levels. In this study, those hepatitis B surface antigens (HBsAg) positive and anti-HCV positive in their peripheral serum were defined as groups infected with HBV and HCV. Liver cirrhosis was diagnosed by pathological examination, and stages of tumor were confirmed according to the TNM staging system.

For survival analysis, all patients were followed and underwent serial monitoring every 2 months for the first 2 years and semiannually thereafter for detection of any recurrence. The last follow-up day was set on August 31, 2012, and survival status was confirmed by means of clinic records and patient or family contact. In this study, the duration of recurrence-free survival (RFS) was defined as from the date of curative treatment to the date of tumor recurrence or last known date alive, whereas the duration of overall survival (OS) was defined as from the date of curative treatment to the date of death or last known date alive. More detailed information was described in our previous studies [19, 20].

2.2. AFB1-DNA Adducts Analysis. Genomic DNA was extracted from HCC tumor tissues and SMMC-7721 cells in a 1.5 mL microcentrifuge tube for deparaffinization and proteinase K digestion, as described by standard procedures (Protocol #BS474, Bio Basic, Inc., Ontario, Canada). DNA was treated according to previously reported procedure, and N-7 adduct was converted to AFB1-FAPy adduct. AFB1-FAPy adduct was quantitated by competitive enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody 6A10 (Novus Biologicals LLC, catalog # NB600-443) [23]. For analysis, AFB1-DNA adduct levels were divided into two groups: low level ($\leq$3.00 μmol/mol DNA) and high level ($\geq$3.01 μmol/mol DNA), according to the average value of AFB1-DNA adduct levels among cases.

2.3. MiR-429 Expression Assay. Quantitative reverse transcription-PCR was used to test miR-429 expression. Total RNA was isolated from tissue or cell cultures, using PureLink RNA Mini Kit (cat# 12183018A, Ambion, USA) according to manufacturer's instructions. For mRNA quantitation, RNA was reversed transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (cat# 4368814, Invitrogen Grand Island, NY, USA) and TaqMan MicroRNA Reverse Transcription Kit (with including miR-429 primer and U6 primer, cat# 4366596, Applied Biosystems (ABI), Carlsbad, CA) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using standard protocols on a Bio-Rad iCycler iQ5 Detection System. Mature miR-429 expression was assessed using TaqMan microRNA assays (cat# 4427975, ABI) with human U6 as the endogenous control. PCR reactions were run in a 5 mL final volume containing 1 × TaqMan Universal Master Mix II (cat# 4440041, ABI), 1 × TaqMan microRNA probes and primers, and about 15 ng of cDNA. Cycling conditions were 95°C for 30 s and 50 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were conducted in triplicate, and controls were performed with no template or no reverse transcription for each gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined for each gene. For tissue samples, the relative amount of miR-429 to U6 was calculated as $2^{-\Delta Ct}$ method, where $\Delta Ct = (Ct_{miR\text{-}429} - Ct_{U6})$. For analysis, miR-429 expression levels were divided into two groups: (1) low expression, $2^{-\Delta Ct} \leq 2$; and (2) high expression, $2^{-\Delta Ct} > 2$, according to the average value among HCC cases. For the relative expression of miR-429 in cancer cells, miR-429 expression was normalized to endogenous controls U6 by the comparative CT method ($2^{-\Delta\Delta Ct}$ method [24]).

2.4. Cell Lines and Culture. The QSG-7701 cells (a kind of peritumoral liver cells) and SMMC-7721 cells (a kind of HCC cancer cells) were obtained from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences. Cells were cultured in DMEM medium (HyClone, Thermo Fisher Scientific (China) CO., Ltd., Shanghai, China) containing high glucose and L-glutamine supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2/100% humidity. All experimental analyses were done with cells in logarithmic growth. Cells were determined to be free of Mycoplasma.

2.5. Cell Transfection and Cell Proliferation and Apoptosis Assay. Cells were transfected with an miR-429 mimic, its inhibitor, its mock, or null control (GenePharma, China) using Lipofectamine 2000 (cat# 11668-027, Invitrogen Grand Island, NY, USA) according to the manufacturer's instructions. The cell proliferation assay was done with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays. Cells were seeded into 96-wells plates for MTT analysis. Twenty-four hours after cell transfections, 20 μL of MTT (5 mg/mL) was added into each well

and incubated for 4 hours. After that, the supernatant was discarded, and then 150 μL dimethyl sulfoxide was added to each well and oscillated for 10 min to dissolve the precipitate. Finally, OD absorbance (at 490 nm) was measured using UV spectrophotometer at 24 hours, 48 hours, and 72 hours after transfection. The assay was performed three times in eight replicates.

In this study, we used flow cytometry technique to elucidate cell apoptosis. Cells were seeded in 6 wells, and the transfections were performed when cells reached 70% confluent. Forty-eight hours after transfection, cells were harvested, washed, and resuspended for cell counting analysis and apoptosis.

2.6. AFB1 Toxicity Analysis. AFB1 toxicity value was evaluated as our previous methods [19]. Briefly, 48 hours after transfections, cells were treated with AFB1 (Sigma) at final concentrations of 24 nM for 1 day, and then the DNA was extracted for AFB1-DNA adduct analysis.

2.7. Statistical Analysis. All analyses were performed with the statistical package for social science (SPSS) version 18 (SPSS Institute, Chicago, IL, USA). MiR-429 expression among different tissues and cells was compared by independent two-sample t-test for two groups or one-way ANOVA with Bonferroni corrections for three or more than three groups. Nonconditional logistic regression was used to evaluate odds ratios (ORs) and 95% confidence intervals (CIs) for the effects of miR-429 expression on the pathological features of HCC. Kaplan-Meier survival analysis with the log-rank test was used to evaluate the relationship between miR-429 expression and HCC prognosis. Hazard ratios (HRs) and 95% CIs for miR-429 expression were calculated from multivariate Cox regression model. In this study, a P value of <0.05 was considered statistically significant.

3. Results

3.1. The Characteristics of HCC Patients. The demographic data of the patients are shown in Table 1. The present study comprised of 138 HCC patients with 125 (90.6%) males and 13 (9.4%) females. The mean age was 46.7 ± 11.7 years. The HBV and HCV infective rates were 84.1% (116 of 138) and 1.4% (2 of 138), respectively. One hundred percent cases featured HCC with I-II TNM stage and received the same curative resection treatment. Most of them had liver cirrhosis and featured AFB1 exposure. In this study, we elucidated AFB1 exposure levels through testing AFB1-DNA adducts of DNA samples from cancerous tissue of the patients and found the mean of 2.98 ± 1.48 μmol/mol DNA. This suggested that higher levels of adducts were in DNA samples from cancer tissues than in those from peripheral blood [20].

3.2. MiR-429 Was Downregulated in HCC Samples and in SMMC-7721 Cells. We analyzed the expression of mature miR-429 and U6 RNA in HCC tumor tissues and adjacent noncancerous tissues and evaluated the significance of differential miR-429 expression by comparing Ct values in these two types of tissues. We observed that 132 patients had significantly higher levels of miR-429 expression in tumour tissues than in nonmalignant adjacent liver tissues. The average expression of miR-429 was significantly higher in HCC tumor samples (TT), when compared with adjacent noncancerous tissues (NT, Figure 1(a)). We also found similar results in the expression analysis of this microRNA in HCC cell lines SMMC-7721 and nontumor liver cell lines QSG-7701 (Figure 1(b)).

Table 1: Characteristics of the patients with HCC.

Characteristics	
Age, yr	
Mean ± SE	46.7 ± 11.7
Range	15–75
Sex	
Man, *n* (%)	125 (90.6)
Female, *n* (%)	13 (9.4)
Ethnicity	
Han, *n* (%)	107 (77.5)
Zhuang, *n* (%)	31 (22.5)
HBV infection	
HBsAg (−), *n* (%)	22 (15.9)
HBsAg (+), *n* (%)	116 (84.1)
HCV infection	
anti-HCV (−), *n* (%)	136 (98.6)
anti-HCV (+), *n* (%)	2 (1.4)
Smoking status	
No, *n* (%)	109 (79.0)
Yes, *n* (%)	29 (21.0)
Drinking status	
No, *n* (%)	110 (79.7)
Yes, *n* (%)	28 (20.3)
AFB1 exposure	
low, *n* (%)	72 (52.2)
High, *n* (%)	66 (47.8)
Liver cirrhosis	
No, *n* (%)	2 (1.4)
Yes, *n* (%)	136 (98.6)
TNM stage	
I, *n* (%)	8 (5.8)
II, *n* (%)	130 (94.2)
Tumor size	
≤5 cm, *n* (%)	68 (49.3)
>5 cm, *n* (%)	70 (50.7)
Total, *n* (%)	138 (100)

3.3. MiR-429 Expression Associated with Tumor Sized and AFB1-DNA Adduct Levels. To investigate the association between miR-429 expression and clinicopathological features of HCC, we divided miR-429 expression in cancerous tissues into two groups: low expression group (relative level ≤ 2) and high expression group (relative level > 2), according to the average expression levels. We next analyzed the distribution

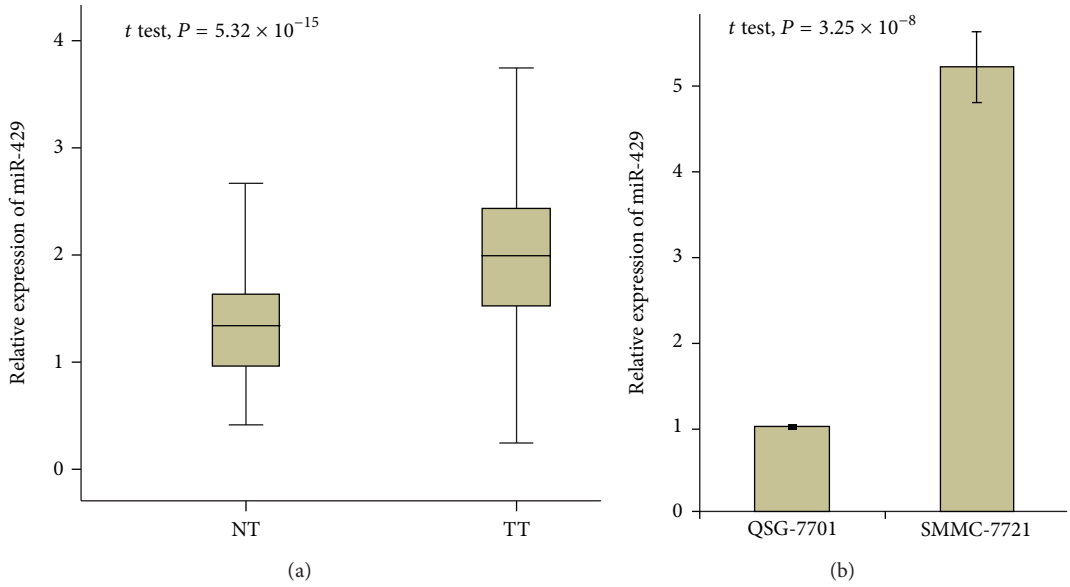

Figure 1: MiR-429 expression correlated with HCC tumorigenesis. (a) MiR-429 expression was evaluated in the tumor tissues, compared with in the adjacent noncancerous tissues. The relative expression of miR-429 is shown as box plots, with horizontal lines representing the median, the bottom, and the top of the boxes representing the 25th and 75th percentiles, respectively, and vertical bars representing the range of data. We compared the difference among groups using the Student t-test. (b) MiR-429 expression was higher in cancer cell line SMMC-7721 than in noncancer cell line QSG-7701.

Table 2: Expression levels of miR-429 and AFB1 exposure levels and tumor size of cases.

	Low expression		High expression		OR (95% CI)[a]	P
	n	%	n	%		
AFB1 exposure						8.88×10^{-3}
Low level	42	64.6	30	41.1	Reference	
High level	23	35.4	43	58.9	2.70 (1.28–5.56)	
Tumor size						3.10×10^{-3}
≤5 cm	41	63.1	27	37.0	Reference	
>5 cm	24	36.9	46	63.0	3.13 (1.47–6.67)	

[a]Adjusted by age, sex, race, HBV and HCV infection status, and smoking and drinking status.

difference of this microRNA expression among different clinicopathological characteristics of cases. Results showed that miR-429 expression levels modified tumor size (OR = 3.13, 95% CI = 1.47–6.67) and AFB1-DNA adduct quantity (OR, 2.70; 95% CI, 1.28–5.56, Table 2) but did not affect other features (data not shown).

3.4. MiR-429 Expression Correlated with HCC Prognosis. During the follow-up period of 138 HCC patients, 47 faced tumor recurrence with 60.1% of the 5-year RFS rate, and 72 died with 62.3% of the five-year OS rate. Kaplan-Meier survival analysis exhibited that patients with high miR-429 expression featured a significantly poorer prognosis than those with low miR-429 expression ($P = 2.45 \times 10^{-8}$ for RFS and $P = 1.28 \times 10^{-9}$ for OS, respectively, Figure 2). Multivariate cox regression analysis (with stepwise forward selection based on likelihood ratio test) was performed to determine whether miR-429 expression was an independent predictor of RFS for patients with HCC. The results showed that miR-429 expression was significantly associated with poorer RFS prognosis (HR = 6.94, 95% CI = 3.19–15.08, $P = 9.94 \times 10^{-7}$, Figure 2(a)). Risk role was also found in the OS analysis; the corresponding HR (95% CI) for high miR-429 expression was 4.64 (2.56–8.41), with a P value 4.11×10^{-7} (Figure 2(b)). Next stratified analysis showed similar risk value (Figure 3). Taken together, these results showed that this microRNA is independent of other clinical covariates and suggested that it could be used as an independent prognostic factor for HCC.

3.5. MiR-429 Expression Modified SMMC-7721 Cell Proliferation. We evaluated the functional role of miR-429 in liver cancer cells by means of measuring cell proliferation in SMMC-7721 cells which were transfected with miR-429 mimic and its inhibitor. In this study, according to the types of mimics transfected, cell lines were divided into four groups: control group (Control, by null mimics), mock group (Mock, by mock mimics), miR-429 group (miR-429, by mature miR-429 mimics), and inhibitor group (Inhibitor, by inhibitor of mature miR-429). MTT assays were next employed to detect the proliferation of SMMC-7721 cell lines. Overexpression of

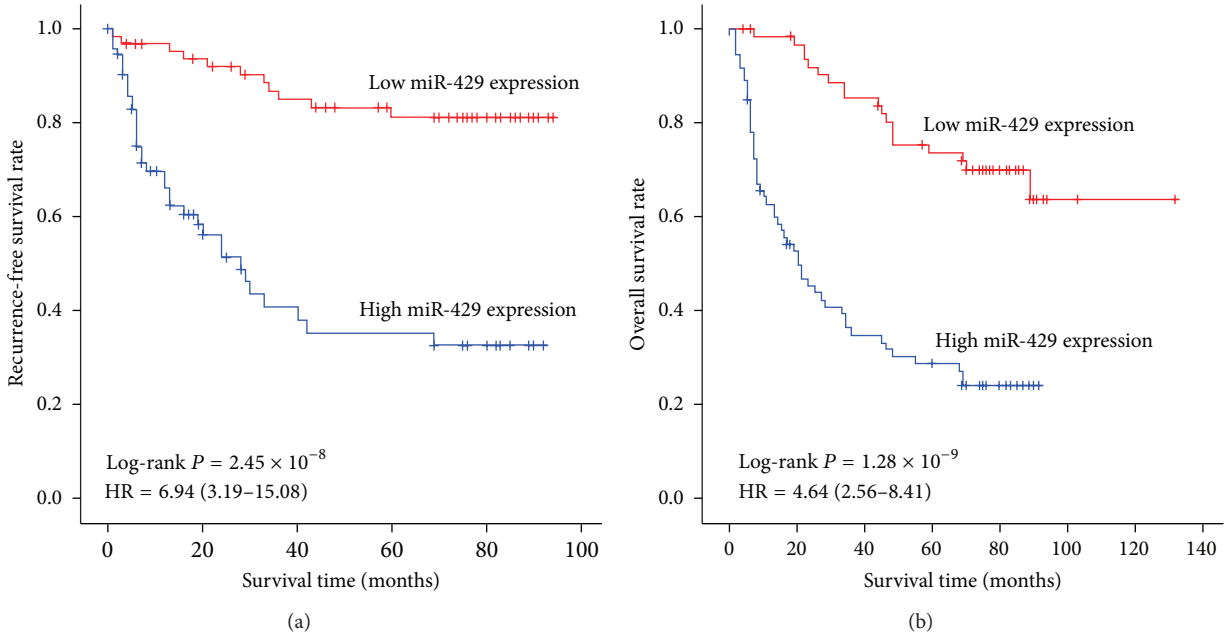

Figure 2: Association between survival and miR-429 expression in 138 HCC cases receiving curative treatment. According to the average expression in cancerous tissues, miR-429 expression was divided into two groups: low expression group (relative level ≤ 2) and high expression group (relative level > 2). MiR-429 expression was associated with tumor reoccurrence-free survival (a) and overall survival (b) of HCC. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. Relative hazard ratio (HR) and corresponding 95% CI of high miR-429 expression (compared with low expression) were calculated using multivariable cox regression model (including all significant variables).

miR-429 in SMMC-7721 cells promoted proliferation, while downregulation of miR-429 in SMMC-7721 cells inhibited cell proliferation. The proliferation of tumor cells in the miR-429 groups, compared with the control group, noticeably increased at 48 h and 72 h ($P < 0.05$, Figure 4(a)). On the other hand, compared with the mock group, the proliferation of tumor cells in the inhibitor groups was inhibited significantly at 48 h and 72 h ($P < 0.05$, Figure 4(a)).

3.6. MiR-429 Expression Modulated SMMC-7721 Cell Apoptosis. We also investigated the function of miR-429 in SMMC-7721 cells through analyzing changes in apoptosis after the liver cancer cells were transfected with miR-429 mimic and its inhibitor. DNA content of transiently microRNA-transfected cells was analyzed by flow cytometry. The early and late apoptosis of SMMC-7721 cell lines in the miR-429 group was significantly inhibited ($P < 0.05$) compared with the control group. Tumor cell apoptosis in the inhibitor group, compared with the mock group, was promoted significantly ($P < 0.05$) (Figure 4(b)).

3.7. MiR-429 Expression Increased AFB1-DNA Adducts in the SMMC-7721 Cells. To explore the effects of miR-429 expression on AFB1-DNA formation, we accomplished a toxin experiment of AFB1 in the SMMC-7721 cells transfected by different mimics. Results showed that group with overexpression of miR-429 had elevated levels of AFB1-DNA adducts (0.78 ± 0.12 nmol/μg DNA) compared with control group (0.39 ± 0.05 nmol/μg DNA, $P < 0.05$, Figure 5). On the other hand, compared with mock group (0.43 ± 0.04 nmol/μg DNA), cells transfected by miR-429 inhibitor featured decreased levels of DNA adducts (0.18 ± 0.02 nmol/μg DNA, $P < 0.05$, Figure 5).

4. Discussion

HCC is the most common histological type of liver cancer in the world [3, 7]. More than 80% of all HCC cases occur in developing countries, and approximately 55% of all cases occur in China (especially in the southeast areas such as Guangxi Zhuang Autonomous Region) [3]. Because of metastasis or other causes, most HCC cases are already in an incurable stage with an extremely poor prognosis at the time of diagnosis [6, 25]. Therefore, new prognosis biomarkers and therapies have been expected, but no remarkable advances have been made in the treatment and prognostic prediction of this malignant tumor [4].

Increasing number of studies has shown that microRNAs may be a type of significant prognosis factor and potential therapeutic target. MicroRNAs are a class of small noncoding single-stranded RNA and typically contain 18–24 nucleotide sequence [26–30]. Originally, they were found as an evolutionarily conserved class of small RNAs which are formed from the sequential processing of primary transcripts by two RNase enzymes, Drosha and Dicer [28–30]. To date, it has been identified that there are more than 1,800 microRNAs in the mammalian genome (miRDatabase). Functionally, microRNAs are involved in regulating gene expression and play a role in a very wide range of physiological processes

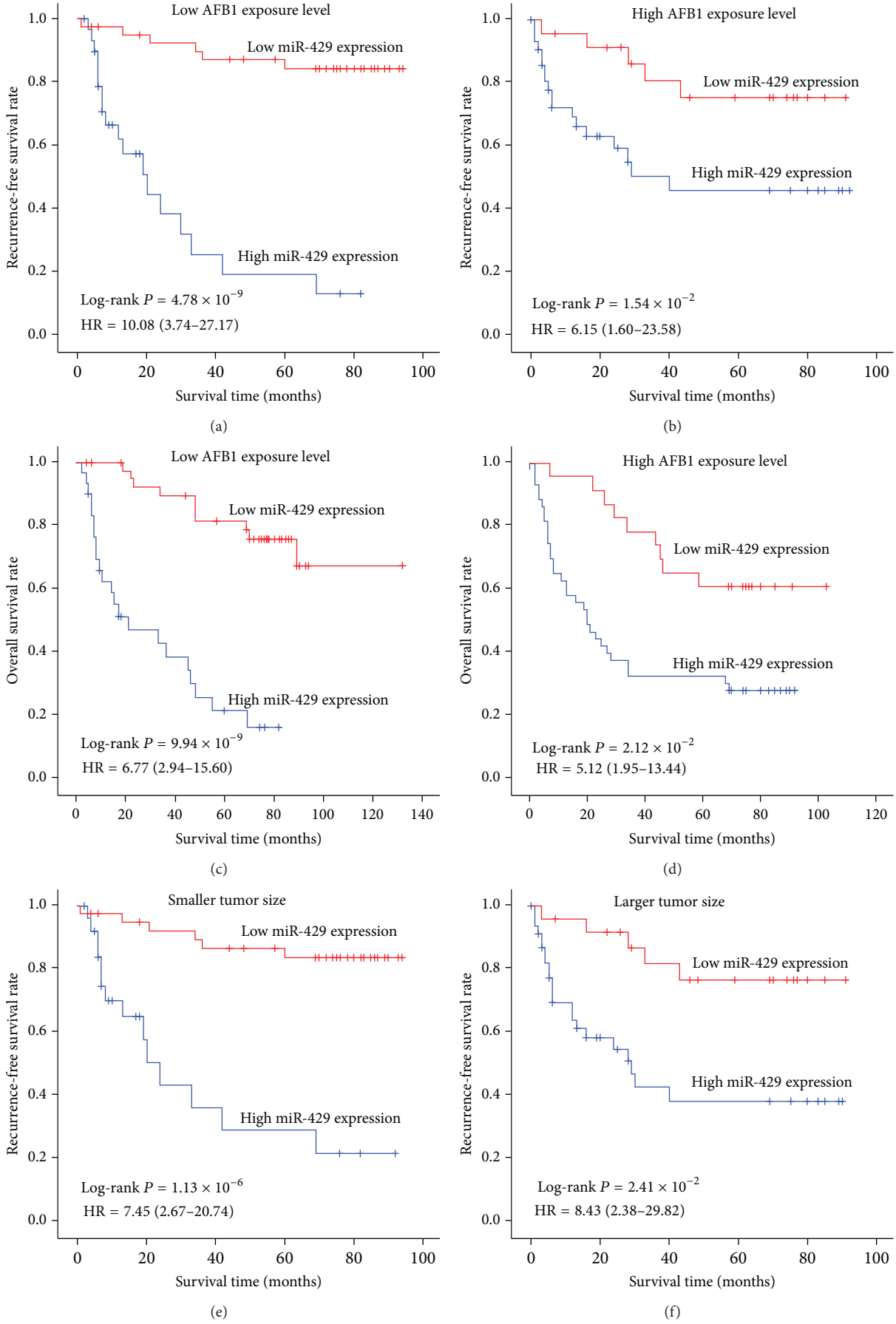

Figure 3: Continued.

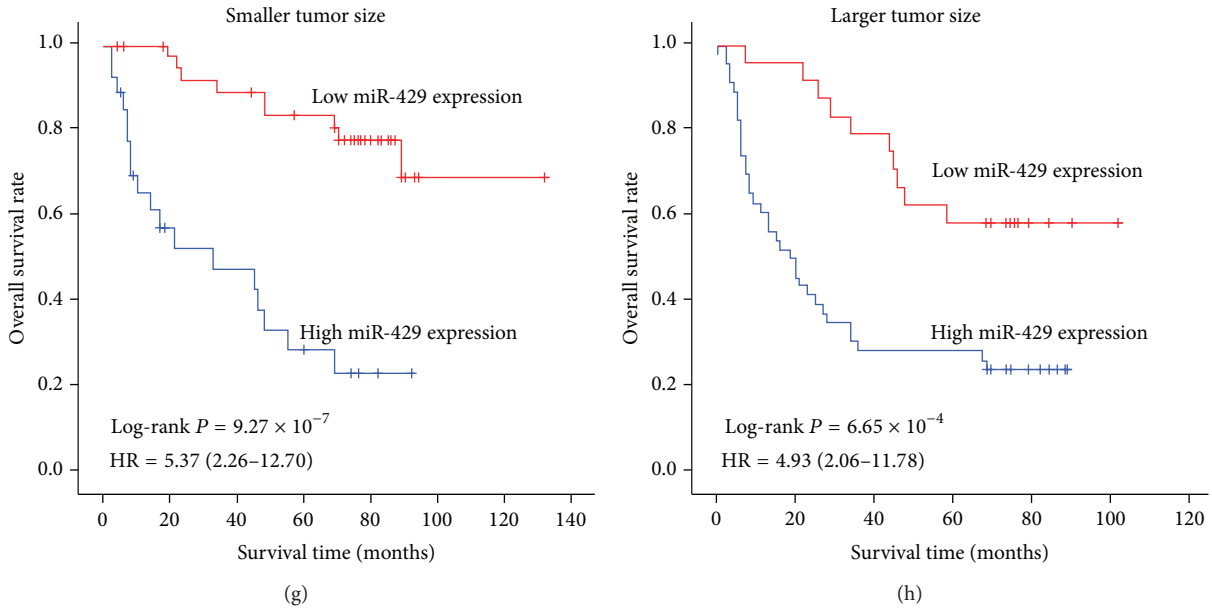

Figure 3: Survival analysis of miR-429 expression in strata of AFB1-DNA adducts and tumor size. ((a)-(b)) Tumor recurrence-free survival (RFS) and miR-429 expression in strata of AFB1-DNA adduct levels. ((c)-(d)) Overall survival (OS) and miR-429 expression in strata of AFB1-DNA adduct levels. ((e)-(f)) RFS and miR-429 expression in strata of tumor size. ((g)-(h)) OS and miR-429 expression in strata of tumor size. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. Relative hazard ratio (HR) and corresponding 95% CI of high miR-429 expression (compared with low expression) were calculated using multivariable cox regression model (including all significant variables).

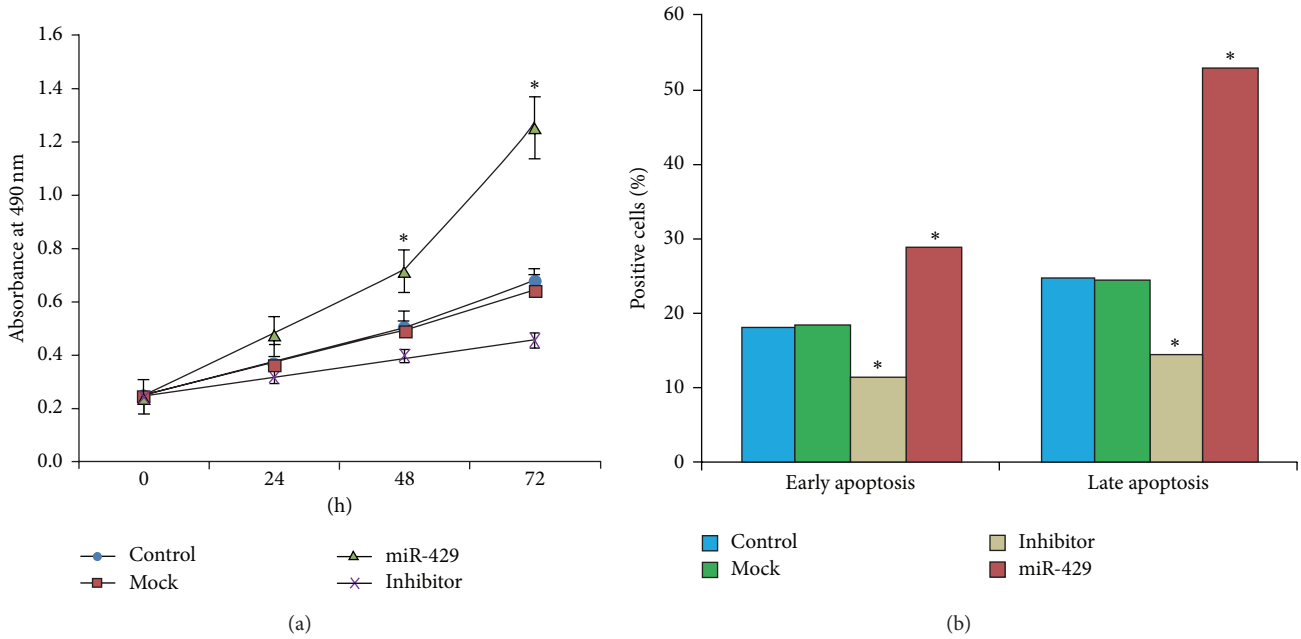

Figure 4: MiR-429 expression modified the proliferation and the apoptosis of HCC cancer cells. According to the types of mimics transfected, cell lines were divided into four groups: control group (Control, by null mimics), mock group (Mock, by mock mimics) miR-429 group (miR-429, by mature miR-429 mimics), and inhibitor group (Inhibitor, by inhibitor of mature miR-429). (a) Association between miR-429 expression and cancer cell proliferation was elucidated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays. (b) Relationship between miR-429 expression and cancer cell apoptosis was evaluated by flow cytometry technique. Data were analyzed using one-way ANOVA with Bonferroni corrections. $^{*}P < 0.05$.

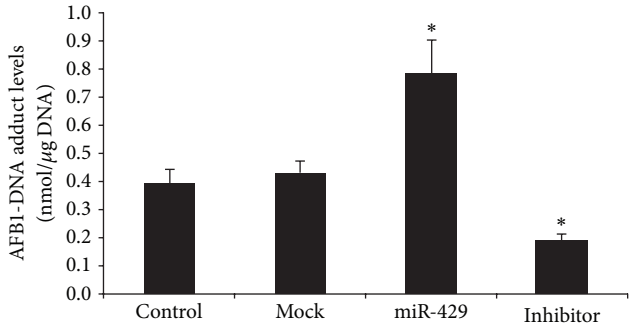

Figure 5: AFB1-DNA adducts formation in AFB1-treated SMMC-7721 cells with overexpression of miR-429 (see Section 2). Levels of AFB1-DNA adducts were tested using comparative ELISA. Data were analyzed from three independent tests using one-way ANOVA with Bonferroni corrections. $^{*}P < 0.05$.

including cell differentiation, cell proliferation, cell apoptosis, physiological timing, metabolism, and hormone secretion [31–33]. Furthermore, increasing evidence has exhibited that this type of small RNAs may play a role in the aetiology and pathogenesis of various cancers by targeting a number of oncogenes or tumour suppressors genes [34]. Recent several reports have shown that the dysregulation of some microRNAs expression may modify the prognosis and the clinicopathological features of tumors such as colon cancer [35, 36], gastric cancer [37, 38], lung cancer [39], and skin cancer [34, 40].

Of particular recent interest is the possible contribution of miR-429 to tumor prognosis and clinicopathological characteristics [13, 15, 17, 18, 41, 42]. MiR-429 is classified as a member of miR-200 family and may play an important role in tumor prognosis. For example, Li et al. [15] investigated the correlation between miR-429 expression and colorectal cancer. They found that miR-429 expression is higher in the cancerous tissues than nontumor tissues. Survival analysis showed that the expression of this microRNA modified colorectal cancer outcome and was an independent prognostic factor for malignant tumor. In this study, we collected 138 HCC tissue samples from Guangxi Zhuang Autonomous Region, a high epidemic area of HCC in China, and explored the possible effects of miR-429 expression on HCC prognosis using TaqMan-PCR technique. We found that these patients having high miR-429 expression in the tumor tissues had a significant poor RFS and OS after curative resection compared with those with low expression of miR-429. Multivariate cox regression analysis exhibited high expression of this microRNA that increased about 6-times tumor reoccurrence risk and 4-times death risk; moreover, this risk did not depend on the clinicopathological change. Taken together, these data suggested that miR-429 expression should be an independent prognostic factor for HCC and that its aberrant expression could be used as a prognostic marker for this tumor. Therefore, it is well known that postoperative adjuvant therapy might significantly improve HCC prognosis.

Recently, Li et al. [15], Sun et al. [13], Liu et al. [17], Hashimoto et al. [16], and Chen et al. [18] have shown that miR-429 expression may modulate the tumorigenesis of gastric cancer and ovarian cancer. To explore whether this microRNA modified liver tumorigenesis, we tested expression difference of miR-429 in different tissue samples and cells and analyzed the effects of this microRNA on cell function in vitro. Our results showed that miR-429 was markedly upregulated in human HCC tumor tissues compared with adjacent noncancerous tissues. This different expression was also confirmed in cell culture in vitro. In the next analysis of cellular function, we only investigated the effects of miR-429 on liver cell proliferation and apoptosis, mainly because of the following reasons. On the one hand, infinite proliferation capacity is a key characteristic of malignant tumors [7]. On the other hand, apoptosis is a major barrier that must be circumvented during tumor development, and evasion of apoptosis is considered to be one of the major hallmarks of tumorigenesis [43]. Reduced rates of apoptosis within the liver tissues are associated with increased risk of HCC [7, 44]. Our results exhibited that the overexpression of miR-429 progressed cell proliferation and inhibited cell apoptosis. On the contrary, the suppression of miR-429 expression hindered cell proliferation and promoted cell apoptosis. These data suggest that this microRNA plays an important role in liver tumorigenesis, and functionally acts as an oncogene in HCC. Supporting our hypothesis that miR-429 has an oncogenic role, recent several studies have exhibited that it can promote the carcinogenesis of pancreatic ductal adenocarcinoma, gastric cancer, and colorectal cancer by targeting EP-300, SOX2, or c-myc [15, 16, 42], while the inhibitors of miR-429 significantly suppressed such tumor cells as of endometrial cancer cells and gastric cancer cell proliferation. However, this microRNA is shown to have a tumor-suppressor function in breast cancer and gastric cancer [13, 17]. The different function of miR-429 in different types of cancer may be because of the differences of cellular context or alternatively the targeted genes.

In the present study, we explored the relationship between miR-429 expression and the clinicopathological features of HCC and found this microRNA only modulated tumor size. These patients with high miR-429 expression would face higher tumor size, suggesting that this microRNA might promote tumor growth and subsequently might play an important role in the carcinogenesis of HCC.

Additionally, we also investigated the association between miR-429 expression and AFB1, mainly because AFB1 is a major carcinogen for liver cancer in China, especially Guangxi Zhuang Autonomous Region [1]. This carcinogen is produced by fungi of the *Aspergillus spp*. and metabolized mainly by cytochrome P450 into the genotoxic metabolic AFB1-exo-8,9-epoxide (AFB1-epoxide). AFB1 epoxide is able to bind to DNA and causes the formation of AFB1-DNA adducts. Increasing evidence has shown that the levels of AFB1-DNA adducts correlate with HCC risk and prognosis, whereas the formation process of AFB1-DNA adducts can be modified by some factors such as detoxifying enzymes and DNA repair enzymes [1, 2, 45], while our present studies exhibited that miR-429 expression promoted AFB1-DNA adducts formation and increased adducts mount in liver cancer tissues. The aforementioned results were furthermore proved in the toxicological analysis of AFB1 in vitro. This is possibly because miR-429 can target some detoxification

enzyme genes and/or DNA repair genes and reduce their detoxification capacity or DNA repair capacity and subsequently increase DNA damage and promote AFB1-DNA adducts formation. These results provided new insights into the mechanism of HCC induced by AFB1.

The present study had several limitations. Only 138 HCC patients were enrolled in the analysis of the clinicopathological characteristics and prognosis. We would like to confirm the findings in a larger liver cancer patient population. Another important limitation is that we did not do migration and invasiveness assays to validate the involvement of miR-429 in tumour migration and invasion.

5. Conclusions

In summary, this study is, to the best of our knowledge, the first report that describes miR-429 expression in liver cancer and its associations with HCC prognosis. Our results showed that miR-429, as an oncogene, was overexpressed in liver cancer tissues and could be considered as a potential prognostic factor for HCC. Furthermore, overexpression of this microRNA promoted proliferation and inhibited apoptosis in liver cancer cells. Therefore, more detailed molecular pathogenesis analysis deserves elucidation based on the results from large samples. Expanding insights into the key role of dysregulated microRNAs involved in liver tumorigenesis will yield important clues for the complicated molecular pathogenesis of HCC and may assist the development of new therapeutic regimens for HCC patients.

Disclosure

All authors declare no commercial association, such as consultancies, stock ownership, or other equity interests or patent licensing arrangements.

Authors' Contribution

Xiao-Ying Huang, Jin-Guang Yao, and Hong-Dong Huang contributed equally to the work.

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Research Article

H-Ras Oncogene Expression and Angiogenesis in Experimental Liver Cirrhosis

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Background. Proto-oncogenes, particularly ras, may not only affect cell proliferation but also contribute to angiogenesis by influencing both proangiogenic and antiangiogenic mediators. The aim of this study was to investigate whether any relationship exists between ras expression and angiogenesis during diethylnitrosamine- (DEN-) induced experimental liver fibrosis. *Materials and Methods*. Liver cirrhosis was induced in rats by intraperitoneal injections of DEN. The animals were sacrificed 2 weeks after the last administrations and a hepatectomy was performed. Masson's trichrome staining was used in the evaluation of the extent of liver fibrosis. The vascular density in portal and periportal areas was assessed by determining the count of CD34 labeled vessel sections. For quantitative evaluation of H-ras expression, in each section positive and negative cells were counted. *Results*. In fibrotic group H-ras expression was higher than that in nonfibrotic group and was more widespread in cirrhotic livers. Friedman's test showed that there was a significant correlation between H-ras expression and VD ($P < 0.01$). *Conclusion*. The results of this descriptive study reveal that H-ras expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis.

1. Background

Proto-oncogenes, particularly ras, may not only affect cell proliferation but also contribute to angiogenesis by influencing both proangiogenic and antiangiogenic mediators [1, 2].

Recently the close association of fibrogenesis and angiogenesis during liver injury has been demonstrated and it has been proposed that its manipulation could be promising in the treatment of chronic liver diseases which respond poorly to conventional therapies [3].

Numerous studies have shown increased ras expression in experimental models and in liver specimens from cirrhotic patients of different etiologies. It has been proposed that increased expression of ras in cirrhotic livers might be associated with inflammation and fibrosis, besides its suggested role in the early step of hepatocarcinogenesis [4]. However the relationship between ras expression and angiogenesis during liver fibrogenesis has not been documented.

Therefore, the aim of this study was to investigate whether any relationship exists between ras expression and angiogenesis during diethylnitrosamine- (DEN-) induced experimental liver fibrosis.

2. Methods

Liver cirrhosis was induced in rats by intraperitoneal injections of DEN (Sigma, Saint-Quentin-Fallavier, France) at 100 mg/kg of body weight ($n = 24$) or 0.9% sodium chloride ($n = 6$) once a week. The injections were applied for 2 ($n = 4$), 4 ($n = 4$), 5 ($n = 4$), 6 ($n = 4$), 8 ($n = 4$), and 10 ($n = 4$) weeks. The rats were sacrificed 2 weeks after the last applications and hepatectomies were performed. Liver tissue samples were fixed in 10% buffered formalin and embedded in paraffin.

Four-micrometer-thick sections were prepared and stained with hematoxylin-eosin and Masson's trichrome

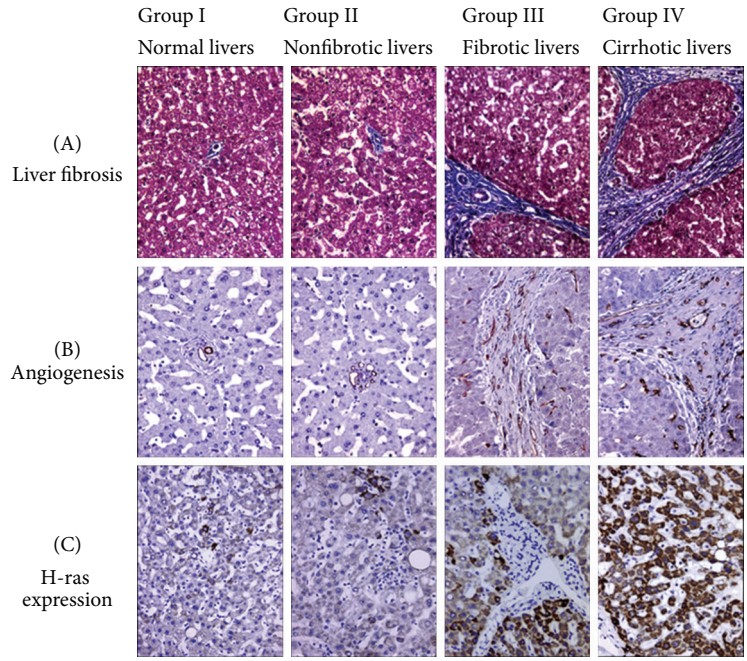

Figure 1: Liver fibrosis (A), angiogenesis (B), and H-ras expression (C) in the study group. In normal livers, the number of CD34 labeled vessels* and H-ras positive cells* is lower when compared to DEN-treated livers. In the latter, their number increases according to the extent of fibrosis (*brown color staining).

Table 1: Distribution of mean, standard deviation (SD), median, and ranges of vascular density (VD) and H-ras expression in normal livers (group I), nonfibrotic (group II), fibrotic (group III), and cirrhotic livers (group IV). The Mann-Whitney *U* test was used.

Group	VD				H-ras (%)			
	Mean ± SD	Median	Range	*P*	Mean ± SD	Median	Range	*P*
I ($n = 6$)	3.1 ± 0.95	3	0–7	>0.05	0.66 ± 0.58	0	0–4	>0.05
II ($n = 8$)	6.82 ± 2.5	7	2–11	<0.05	13 ± 6.08	12	3–30	<0.05
III ($n = 8$)	11.3 ± 2.4	10	9–16	<0.05	19.6 ± 6.39	22	10–30	<0.05
IV ($n = 8$)	15.90 ± 3.7	16	10–22	<0.05	25.87 ± 7.28	26	12–30	<0.05

for the histopathological assessment and the evaluation of the extent of liver fibrosis. Immunolabeling was performed using monoclonal antibodies directed anti-rat CD34 (dilution: 1 : 500, Santa Cruz, CA, USA) and H-ras (dilution: 1 : 200, Santa Cruz, CA, USA). An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit, Santa Cruz, CA, USA) was used for labeling.

The vascular density in portal and periportal areas was assessed by determining the count of CD34 labeled vessel sections at higher magnification (×400), with the use of an ocular grid subdivided into 100 areas. For each subject vascular density (VD) was noted.

For quantitative evaluation of H-ras expression, in each section positive and negative cells were counted in systematically randomly selected 10 to 15 microscopic fields by using an ocular grid at high magnification (×400). The positive staining was calculated as the percentage of positive cells to total number of counted cells. Positive cells touching the left and lower edges of the grid were not included.

All analysis was performed by using Statistical Package for Social Science (SPSS 15.0 for Windows, USA). The Mann-Whitney *U* test was used to establish the difference between groups. Friedman's test was used to determine the relationship among quantitative parameters. Data were expressed as mean ± SD and $P < 0.05$ was considered significant.

3. Results

In this study, fibrous septa formation was detected after 5 weeks and the liver was cirrhotic in all cases after 8 weeks. In the control group any fibrosis was not detected. In respect of the grade of fibrosis, cases were divided into the following groups: group I: normal livers, group II: nonfibrotic livers (2 and 4 weeks), group III: fibrotic livers (5 and 6 weeks), and group IV: cirrhotic livers (8 and 10 weeks) (Figure 1 and Table 1).

While in control (group I) CD34 staining was restricted to the endothelium of portal vessels, numerous CD34-labeled

vessels were detected in fibrotic and cirrhotic livers (Figure 1). The latter CD34 staining revealed a dense vascular plexus surrounding the cirrhotic nodules. In nonfibrotic livers (group II) CD34 expression was noted in a few vascular structures around portal areas. Parallel to these findings, VD values were increased together with the progression of fibrosis (Figure 1). Groups II, III, and IV had higher VD than the control group ($P < 0.05$). The difference among VD values of these groups was also statistically significant ($P < 0.05$) (Figure 1 and Table 1).

H-ras expression was observed in the cytoplasm of the hepatocytes. In normal livers (group I), the expression was restricted to a few periportal hepatocytes. However in DEN-treated rats H-ras expression displayed a heterogeneous distribution. In fibrotic group (group III) H-ras expression was higher than that in group II and was more widespread in cirrhotic livers (group IV) (Figure 1). The expressions of H-ras in DEN-treated rat groups were significantly different from each other ($P < 0.05$) (Figure 1 and Table 1). Moreover, Friedman's test showed that there was a significant correlation between H-ras expression and VD ($P < 0.01$).

4. Conclusion

The results of this descriptive study reveal that H-ras expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis.

Our data suggest that H-ras might contribute to the wound healing response to liver injury not only as a strong activator of hepatic stellate cells leading to fibrosis but also as an inducer of angiogenesis.

In the light of these observations, it would be of interest to evaluate the mechanism triggered by H-ras in hepatic angiogenesis with further experimental models, in order to completely clarify if the use of ras inhibitors would be beneficial in multitargeted treatment of fibrogenesis in chronic inflammatory liver diseases ending with cirrhosis.

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