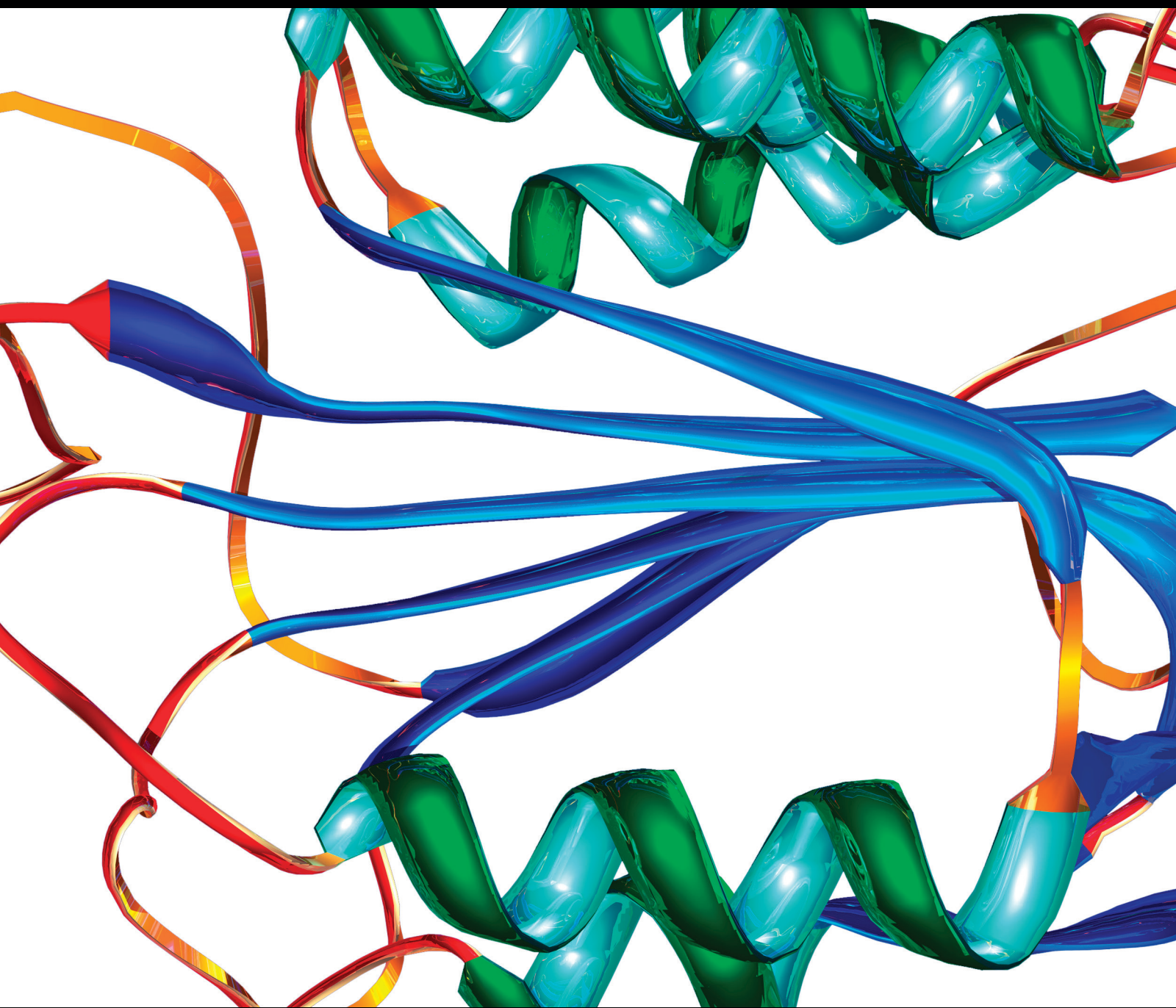
Disease Markers

Developmental Gene Markers in Tumor Pathogenesis and Progression

Special Issue Editor in Chief: Monica Cantile
Guest Editors: Giuseppe Palmieri and Gerardo Botti



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Editorial Board

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Editorial

Developmental Gene Markers in Tumor Pathogenesis and Progression

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Aberrant activity of the genes that regulate cell differentiation and morphogenesis during embryonic development appears to be associated with specific oncogenic processes, from the control of cell growth, proliferation, and apoptosis to cell invasion and epithelial mesenchymal transition.

In this context, one of the most striking examples is represented by the homeobox gene family, a superfamily of transcription factors, mostly involved in the control of the identity of various regions along the body axis, from the branchial area to the tail. In particular, the deregulation of Class I Homeobox genes (HOX genes) is described as strongly associated with neoplastic transformation and disease progression in several human cancers.

The preparation of this special issue resulted in a series of 8 articles that have highlighted the deregulation of different developmental genes in tumor pathogenesis and progression. Several molecules, with different cellular functions, have been analyzed such as membrane receptors (FJX1 and SDC1), adhesion molecules (SALM3), and transcription factor (TFAP4).

Four-jointed Box 1 (FJX1) acts as a receptor for a signaling pathway that regulates growth, gene expression, and planar cell polarity. In fetal and early postnatal brain, it is expressed mainly in the primordia of layered telencephalic structures and in the superior colliculus. S. J. Chai et al. showed that the overexpression of FJX1 contributes to a more aggressive phenotype of nasopharyngeal carcinoma cells suggesting its role as a therapeutic target.

Latent transforming growth factor-beta-binding protein, LTBP2, is an integral component of elastin-containing microfibrils involved in the developing lung, pericardium, epicardium, and heart valves. Y. Huang et al. analyzed LTBP2 expression in colorectal cancer (CRC) patients suggesting that it may act as an oncogene in the development of CRC and in predicting CRC prognosis.

Synaptic adhesion-like molecule 3, SALM3, is expressed in telencephalic and diencephalic vesicles at embryonic day (E) 11.5 to E17.5. Y. Liu et al. showed that SALM3 expression in tumor cells or stroma is an independent prognostic factor in the overall survival rate of gastric cancer patients.

The transcription factor AP4, TFAP4, belonging to the basic helix-loop-helix-zipper (bHLH-ZIP) family is involved in cell maintenance in a proliferative, progenitor-like state by blocking p21 expression. T. Huang et al. described that TFAP4 is able to promote the invasion and metastasis by inducing epithelial-mesenchymal transition (EMT) and regulating MMP-9 expression via activating the PI3K/AKT signaling pathway in hepatocellular carcinoma cells.

Axis inhibitor 1, AXIN1, is involved in both positive and negative regulation of Wnt-beta-catenin signaling during embryonic development. Q. Li et al. analyzed three AXIN1 gene polymorphisms showing their implication in bladder cancer (BC) risk and suggesting new potential forecasting factors for prognosis of BC patients.

Syndecan 1, SDC1, is an integral membrane protein acting as a receptor for the extracellular matrix and involved

in the maturation and release of B lymphocytes. K. Li et al. highlighted that the loss of SDC1 plays an important role in colorectal cancer malignant progression.

Finally, the contribution of DNA methylation status of several biomarkers has also been highlighted. M. Shan et al. showed a high promoter methylation frequency of homeobox genes HOXA11 and HOXD13 in breast cancer tissues.

Z. Ma et al. performed a review article in which they described the role of different methylated biomarkers including tachykinin-1 (TAC1), somatostatin (SST), and runt-related transcription factor 3 (RUNX3) in colorectal cancer patient diagnosis and monitoring.

In conclusion, this special issue collecting novel findings about the role of several development biomarkers will strengthen the hypothesis that the deregulation of genes involved in embryonic development could play a fundamental role in the pathogenic mechanisms of tumor diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

TFAP4 Promotes Hepatocellular Carcinoma Invasion and Metastasis via Activating the PI3K/AKT Signaling Pathway

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Transcription factor activating enhancer binding protein 4 (TFAP4) is established as a regulator of human cancer genesis and progression. Overexpression of TFAP4 indicates poor prognosis in various malignancies. The current study was performed to quantify TFAP4 expression as well as to further determine its potential prognostic value and functional role in patients with hepatocellular carcinoma (HCC). We identified that the expression of TFAP4 mRNA in 369 tumor tissues was higher than that in 160 normal liver tissues. Upregulated TFAP4 expressions were discovered in HCC cell lines compared to the healthy liver cell line, and similarly, the levels of TFAP4 were higher in tumor tissues than its expression in paratumor tissues. High mRNA and protein expression of TFAP4 was associated with worse overall survival (OS) and disease-free survival (DFS). Additionally, TFAP4 expression emerged as a risk factor independently affecting both OS and DFS of HCC patients. Functional studies demonstrated that TFAP4 increased HCC cell migration and invasion. Further investigations found that TFAP4 promotes invasion and metastasis by inducing epithelial-mesenchymal transition (EMT) and regulating MMP-9 expression via activating the PI3K/AKT signaling pathway in HCC. In conclusion, our study demonstrated that TFAP4 is a valuable prognostic biomarker in determining the likelihood of tumor metastasis and recurrence, as well as the long-term survival rates of HCC patients. Exploring the regulatory mechanism of TFAP4 will also contribute to the development of new prevention and treatment strategies for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most important type of liver cancer [1] and is responsible for being the 2nd most frequently encountered cause of global cancer-associated mortalities [2]. Surgical resection of the affected liver lobes is an effective treatment modality for early stage HCC, and over the years, prognosis has improved considerably with the continued improvement of operative skills and instruments [3]. Despite this, patients with HCC who have undergone surgical resection do not possess favorable long-term survival rates due to the risks of metastasis and recurrence. Although several tumor biomarkers have been investigated as prognostic modalities in candidates for surgical resection, predictive abilities of these markers are not optimal [4–8]. Therefore, it is essential to identify novel tumor biomarkers, which may provide suggestions for the auxiliary diagnosis and predict clinical outcome. On this basis, further study of its molecular mechanism of function may provide new ideas for the treatment of HCC.

The basic helix-loop-helix leucine-zipper (bHLH-LZ) family is known to participate in regulating cell proliferation and differentiation [9]. The transcription factor activating enhancer binding protein 4 (TFAP4) is a member of this family and has been found to be widely involved in the proliferation, differentiation, metastasis, angiogenesis, and other biological regulatory functions of malignancies [9–11]. Recently, TFAP4 overexpression was reported to

confer worse prognosis in various malignancies, such as gastric cancer [12], colorectal cancer [13], prostate cancer [14], and non-small-cell lung carcinoma [15]. Jackstadt et al. [16] also found that TFAP4 can enhance migration and metastasis of tumor cells by activating epithelial-mesenchymal transition (EMT). However, the studies on TFAP4 have rarely been reported in HCC. Although Song et al. [17] found that TFAP4 can enhance tumor-forming ability by activating the Wnt/β-catenin pathway in HCC, the effect of TFAP4 on the invasion and metastasis of HCC remains to be studied.

In the present study, we sought to determine TFAP4 expression in HCC cell lines and tissues and performed further analyses to determine its potential correlations with clinicopathological characteristics as well as its ability to prognosticate HCC patients receiving surgical resection. Furthermore, we preliminarily investigated the effect of TFAP4 on HCC invasion and metastasis as well as its possible mechanism. We found that TFAP4 can function as a useful prognostic biomarker and it may promote invasion and metastasis via activating the PI3K/AKT signaling pathway in HCC.

2. Material and Methods

2.1. Gene Expression Profiling Interactive Analysis. The website of gene expression profiling interaction analysis (GEPIA, http://gepia.cancer-pku.cn/) was used to analyze the TFAP4 mRNA expression in 369 HCC tissues and 160 normal liver tissues derived from the TCGA database and the GTEx database. 362 HCC patients derived from the TCGA database were used to investigate the relationship between prognosis and mRNA expression levels of TFAP4. During the analysis, patients were divided into two groups (high TFAP4 and low TFAP4) according to the median expression of TFAP4. Overall survival and disease-free survival of the two groups were compared. In addition, HCC patients derived from the TCGA database were used to analyze the correlations between the expression of TFAP4 and the expression of EMT markers (N-cadherin, Vimentin), related transcription factors (Snail, Slug, ZEB1, and ZEB2), and MMP-9.

2.2. Study Cohort and Follow-Up. 217 patients with HCC who received surgical tumor resection without chemotherapy in two medical centers (Guangzhou, China) were enrolled. 101 patients performed the operation in the First Affiliated Hospital of Sun Yat-sen University, and 116 patients performed the operation in the Sun Yat-sen University Cancer Center from 2007 to 2014. In addition, four different HCC cell lines and one healthy liver cell line as well as 10 fresh HCC tumor samples paired with their paratumor tissues were subjected to western blotting and qRT-PCR to determine their respective TFAP4 expression levels. All the patients were diagnosed with HCC based on the World Health Organization criteria. Tumor stage was determined with the tumor-node-metastasis (TNM) as well as the Barcelona Clinic Liver Cancer (BCLC) classification. The Edmondson-Steiner grading system was used to classify the histologic grade of tumor. All patients provided written informed consent upon enrollment into this study which was preapproved by both medical institutions' Ethics Committee.

All patients received once-monthly follow-up for the first six months after the operation and once every three months after. The endpoint of this study was December 31, 2014. Tumor recurrence and metastasis were confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). Overall survival (OS) was determined to be the duration between surgical resection and death. Disease-free survival (DFS) represented the duration between surgical resection to recurrence or metastasis.

2.3. Immunohistochemistry. In brief, 4 μm thick tissue sections derived from 217 adult patients were immersed in xylene to remove paraffin before being rehydrated in different ethanol gradations. Following this, sections were autoclaved for 5 min in 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) in order to retrieve antigens. Endogenous peroxidase activity was blocked by immersing sections for 20 min in 3% hydrogen peroxide at room temperature. Next, sections were subjected to overnight incubation with TFAP4 antibody (1 : 50, ab28512, Abcam, Cambridge, UK) at 4°C. The following day, sections were washed and reincubated with a secondary antibody for 30 min at 37°C. Final staining was done with hematoxylin, and sections were then dehydrated and mounted.

2.4. Evaluation of Immunostaining. Scoring for staining intensity in each case was based on the following scale: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The proportion of positively stained cells was scored as follows: 1, 0-25%; 2, 25-50%; 3, 50-75%; and 4, 75-100%. The immunoreactive score (IRS) was determined by the sum of the two parameters. Patients were divided into two groups: high (IRS $\geq$ 4) and low (IRS $<$ 4) TFAP4 expression, with an IRS score of 4 being the median of the total score. All sections were scored independently by two pathologists with more than 10 years of working experience who were blinded to patients' clinical outcome and clinicopathological parameters. Five randomized microscopic fields were used to calculate the average score in each section.

2.5. Cell Culture. Human hepatocellular carcinoma (HCC) cell lines (Huh7, MHCC-97H, MHCC-97L, and PLC/PRF/5) and LO2, an immortalized liver cell line, were procured from Cell Bank (Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) was used as a culture medium for cells. All the medium was supplemented with 10% fetal bovine serum. Cells were cultured in an incubator with 5% atmospheric CO_2 at 37°C.

2.6. Quantitative Real-Time PCR. Total RNA was extracted from fresh tissues using the TRIzol agent (Takara Bio, Otsu, Japan). cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara Bio, Otsu, Japan). The real-time quantification PCR was performed using the SYBR Premix Ex Taq Kit (Takara Bio, Otsu, Japan) according to the manufacturers' instructions. The primers used in real-time PCR were as follows: TFAP4 forward primer 5′-GTGCCCACTCAGA

AGGTGC-3′ and reverse primer 5′-GGCTACAGAGCCCT CCTATCA-3′; GAPDH forward primer 5′-GGAGCGAGA TCCCTCCAAAAT-3′ and reverse primer 5′-GGCTGTTG TCATACTTCTCATGG-3′. The cycling parameters were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Each sample was detected in 3 duplicate wells. GAPDH was used as an internal control. The relative expression of TFAP4 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Lentivirus-Mediated Plasmid Transfection. The Lenti-TFAP4 expression vector and Lenti-shTFAP4 vector (targeting sequences : 5′-CCTCGGTCATCAACTCTGTTT-3'; control sequences: 5′-TTCTCCGAACGTGTCACGT-3') as well as their respective control vectors were constructed, verified, and purified by Gene Chem (Shanghai, China). MHCC-97L cells were cultured in 24-well plates (about 50-60% confluence) and transfected with Lenti-TFAP4 expression vector and its control vector according to the manufacturer. MHCC-97H cells were transfected with Lenti-shTFAP4 vector and its control vector. Cells were screened and cultured in the medium with 10% FBS and 8 μg/ml polybrene (Sigma, USA). Western blot was used to detect the transfection rate.

2.8. HCC Cells Treated with PI3K/AKT Signaling Pathway Inhibitors. MHCC-97L-TFAP4 cells and MHCC-97L-Control cells were cultured in the six-well plates to 50-60% confluence. PI3K/AKT pathway inhibitor LY294002 with a final concentration of 30 μM was added to the MHCC-97L-TFAP4 cells. The other two groups of cells (MHCC-97L-TFAP4 cells, MHCC-97L-Control cells) were added an equal volume of DMSO. All cells were cultured in an incubator with 5% CO_2 at 37°C. After 48 hours, cells were used for Transwell migration and invasion experiments as well as western blot.

2.9. Western Blotting. Cells and fresh tissues were lysed using lysis buffer (Beyotime, Shanghai, China) that contained protease inhibitors after being subjected to washing with cold phosphate-buffered saline (PBS) thrice based on manufacturer's instructions. The BCA method was performed to quantify the protein concentration, with 10% SDS-PGE gel used to separate equal amounts of protein from each other. Subsequently, proteins were transferred onto 0.22 μm polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA). Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20 was used to block nonspecific antigens for 90 min at room temperature. After that, membranes were incubated overnight at 4°C with primary antibodies as follows: TFAP4 antibody (1 : 1000, ab28512, Abcam, Cambridge, UK), E-cadherin, N-cadherin, Vimentin (1 : 1000, EMT Antibody Sampler Kit #9782, CST, MA), p-AKT^{Ser473} (1 : 1000, #4060, CST, MA), p-$GSK3\beta^{Ser9}$ (1 : 1000, #5558, CST, MA), GSK-3β (1 : 1000, #12456, CST, MA), MMP-9 (1 : 1000, #13667, CST, MA), p-NF-κB^{P65} (1 : 1000, #3033, CST, MA), and GAPDH antibody (1 : 1000, #5174, CST, MA). The next day, membranes underwent three repeated washings with TBST and were incubated again with secondary antibodies (Cell Signaling Technology, MA) at room temperature for 60 min. After 3 final washings with TBST, an enhanced chemiluminescence (ECL) detection system (Merck Millipore, MA, USA) was used to detect protein expressions.

2.10. Transwell Invasion and Migration Assays. Transwell invasion assay was performed using 8 μm pore chamber inserts (Corning, USA) coated with Matrigel (BD Biosciences, USA) in 24-well plates. 5×10^4 cells suspended in 300 μl DMEM without FBS were seeded in the upper chamber. The lower chamber was added with 700 μl DMEM that contained 5% FBS. After being incubated at 37°C for 36 hours, the cells that had invaded to the lower surface of the chamber were stained by 4% paraformaldehyde. The invading cells were stained with 0.1% crystal violet and counted under a microscope on five random fields. Similarly, the migration assay was performed in the same way without Matrigel.

2.11. Statistical Analysis. Experiments were performed at least three times and representative results were presented. Comparisons of quantitative data were analyzed by the Student t-test between two groups. Data were presented as the mean ± SD. Associations between two gene expressions were analyzed by Spearman's correlation test. The correlations between TFAP4 and clinicopathological characteristics were analyzed with Fisher's exact test and the chi-squared test. The Kaplan-Meier method and the log-rank test were used to analyze the OS and DFS. Univariate and multivariate Cox proportional hazards regressions were carried out to determine factors related to survival. Statistically significant differences were determined when $P < 0.05$.

3. Results

3.1. Overexpression of TFAP4 in HCC Tissues and Cells. The website of gene expression profiling interaction analysis (GEPIA) was used to detect the TFAP4 mRNA expression in 369 HCC tissues and 160 normal liver tissues derived from the TCGA database and the GTEx database. The results showed that the expression of TFAP4 mRNA expression in HCC tissues was higher than that in normal liver tissues (Figure 1(a)). Analysis of 10 human fresh HCC samples using qRT-PCR and western blot revealed that tumor tissues possessed higher TFAP4 mRNA and protein expression levels compared to paratumor tissues (Figures 1(b) and 1(c)). Western blot was employed to quantify TFAPA4 protein expressions in four HCC cell lines and a control cell line (LO2). The HCC cell lines showed a higher expression of TFAP4 than the control cell line (Figure 1(d)).

3.2. Correlation of TFAP4 Expression with Clinicopathological Parameters and Prognosis in HCC. To evaluate the potential ability of TFAP4 to prognosticate HCC patients, 362 patients derived from the TCGA database were subjected to survival analysis. Patients that possessed higher TFAP4 mRNA expressions were found to have worse overall survival rates ($P = 0.035$) and poorer disease-free survival rates ($P = 0.00067$) (Figures 2(b) and 2(c)). To verify these results, TFAP4 expression in 217 HCC tissue samples was further

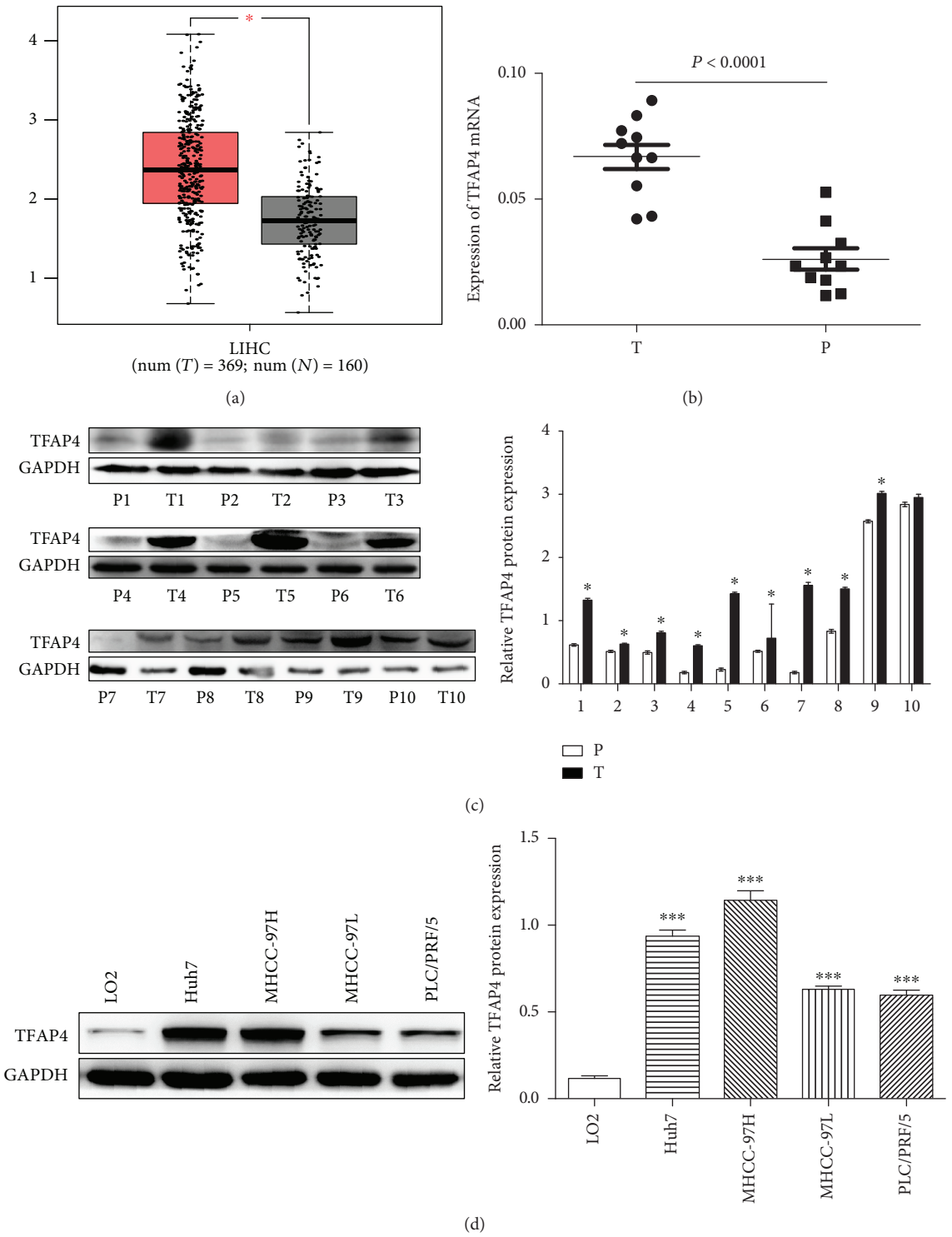

Figure 1: Overexpression of TFAP4 in HCC. (a) GEPIA analyzed the TFAP4 mRNA expression in HCC tissues and normal liver tissues. (b) TFAP4 mRNA expression measured in 10 HCC tumor tissues as well as their adjacent tissues. (c) TFAP4 protein expression measured in 10 HCC tumor tissues as well as their adjacent tissues. (d) TFAP4 protein expression measured in HCC cell lines. HCC: hepatocellular carcinoma; T: tumor tissue; P: paratumor tissue; $^{*}P < 0.05$; $^{***}P < 0.001$.

explored with immunohistochemical staining. TFAP4 staining was primarily observed in the nuclei of tumor cells (Figure 2(a)). Overall, TFAP4 expression was found to be positive in 65.0% ($n = 141$) of the 217 HCC patients. 107 cases showed high levels (IRS ≥ 4) of TFAP4 expression, while 110 cases had low levels (IRS < 4) of TFAP4 expression. Patients that possessed higher TFAP4 expressions were found to have worse overall survival rates ($P = 0.0001$) and

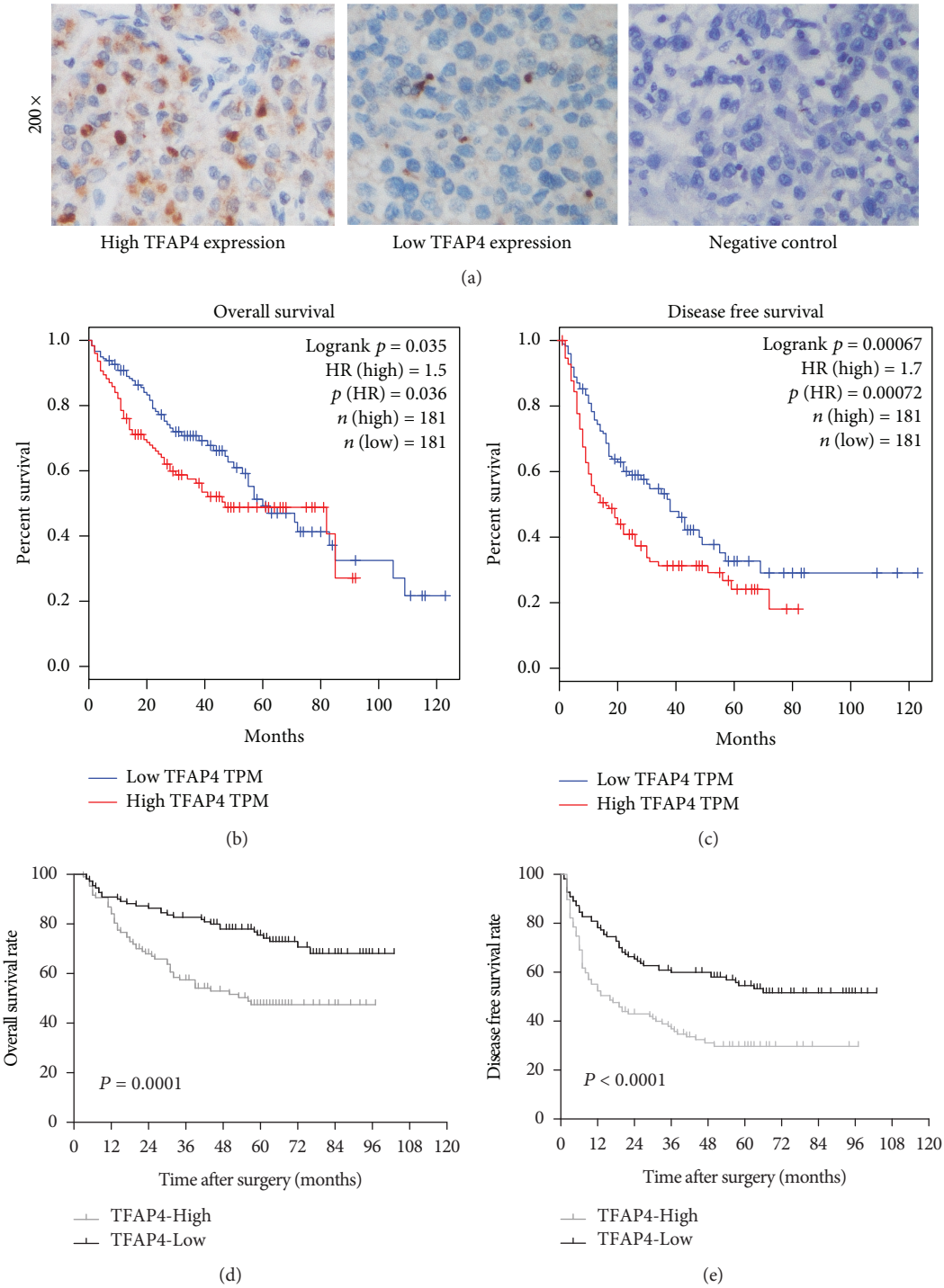

Figure 2: Relationship between TFAP4 expression and prognosis in HCC patients. (a) Immunohistochemical staining in HCC tissues (original magnification ×200). (b) High TFAP4 mRNA expression conferred worse overall survival. (c) High TFAP4 mRNA expression conferred worse disease-free survival. (d) High TFAP4 protein expression conferred worse overall survival. (e) High TFAP4 protein expression conferred worse disease-free survival.

poorer disease-free survival rates ($P < 0.0001$) (Figures 2(d) and 2(e)). Table 1 depicts the correlation between clinicopathological parameters and TFAP4 expression.

Univariate Cox regression analysis revealed that vascular invasion, tumor size, number of tumors, BCLC stage, TNM stage, and TFAP4 expression were all strongly correlated with OS rates (Table 2), while gender, vascular invasion, tumor size, number of tumors, AFP, BCLC stage, TNM stage, and TFAP4 expression were strongly correlated with DFS (Table 3). To further determine if TFAP4 was an independent prognostic factor, several potential clinicopathological features with statistical significance ($P < 0.05$) were subjected

Table 1: Correlation between clinicopathological parameters and TFAP4 expression in HCC patients.

Clinicopathological parameters	Cases (n)	TFAP4 expression		P value
		High	Low	
Age (year)				
<60	176	85	91	0.536
≥60	41	22	19	
Gender				
Male	194	100	94	0.056
Female	23	7	16	
HBsAg				
Positive	183	92	91	0.510
Negative	34	15	19	
AFP (μg/l)				
<400	135	65	70	0.661
≥400	82	42	40	
Tumor size (maximal diameter)				
<5cm	107	52	55	0.836
≥5cm	110	55	55	
Number of tumors				
Solitary	181	87	94	0.412
Multiple	36	20	16	
Encapsulation				
Destructed	74	40	34	0.315
Intact	143	67	76	
Vascular invasion				
Yes	191	17	9	0.081
No	26	90	101	
TNM stage				
I-II	184	92	92	0.631
III	33	15	18	
BCLC stage				
0-A	177	84	93	0.251
B	40	23	17	
Differentiated				
I-II	173	88	85	0.363
III-IV	44	19	25	
Micrometastasis				
Yes	16	10	6	0.273
No	201	97	104	

to further multivariate analysis. Cox proportional hazards regression revealed tumor sizes, TNM stage, and TFAP4 expression to be risk factors that independently determine OS rates (Table 2). Meanwhile, tumor sizes, the presence of vascular invasion, and TFAP4 expression were found to be risk factors that independently affect DFS (Table 3). TFAP4 was found to be an independent prognostic indicator for OS and DFS in HCC patients (HR, 2.712; 95% CI, 1.705-4.314; HR, 2.086; 95% CI, 1.450-3.001, respectively).

HR: hazard ratio; OS: overall survival; CI: confidence interval.

HR: hazard ratio; DFS: disease-free survival; CI: confidence interval.

3.3. TFAP4 Promotes the Invasion and Migration of HCC Cells. According to the expression of TFAP4 in HCC cell lines, MHCC-97H cells and MHCC-97L cells were selected to construct stable silencing and overexpressing TFAP4 cell lines, respectively. Western blot results showed that the protein expression level of TFAP4 in the MHCC-97L-TFAP4 cells was significantly higher than that in the MHCC-97L-Control cells. On the contrary, the protein expression level of TFAP4 in the MHCC-97H-shTFAP4 cells was significantly lower than that in the MHCC-97H-shControl cells (Figure 3(a)). Transwell assay demonstrated that the knockdown of TFAP4 repressed cell migration and invasion in MHCC-97H cells (Figure 3(b)). On the contrary, overexpression of TFAP4 increased cell migration and invasion in MHCC-97L cells (Figure 3(c)). These results indicated that TFAP4 could promote the invasion and metastasis of HCC cells.

To explore the possible mechanism of TFAP4 promoting invasion and metastasis of HCC cells, we carried out further experiments. The results of GEPIA analysis showed that the expression of TFAP4 was positively correlated with the expression of EMT markers (N-cadherin, Vimentin), related transcription factors (Snail, Slug, ZEB1, and ZEB2), and MMP-9 (Figures 4(a)–4(g)). Western blot results also showed that the expression of the epithelial marker E-cadherin was increased and the expression of mesenchymal markers (N-cadherin, Vimentin) and MMP-9 were decreased in the MHCC-97H-shTFAP4 cells compared with its control cells. On the contrary, the expression of the epithelial marker E-cadherin was repressed and the expression of mesenchymal markers (N-cadherin, Vimentin) and MMP-9 were increased in the MHCC-97L-TFAP4 cells compared with its control cells (Figures 4(h) and 4(i)). These results indicated that TFAP4 could induce EMT and promote the expression of MMP-9.

We then explored the possible mechanism of TFAP4 inducing EMT and promoting MMP-9 expression. Western blot results showed that the expressions of p-AKT^{Ser473}, p-GSK3β^{Ser-9}, and p-NF-κB^{P65} were decreased in the MHCC-97H-shTFAP4 cells compared with its control cells. On the contrary, the expressions of p-AKT^{Ser473}, p-GSK3β^{Ser-9}, and p-NF-κB^{P65} were increased in the MHCC-97L-TFAP4 cells compared with its control cells (Figure 5(a)). These results suggested that TFAP4 could activate the PI3K/AKT signaling pathway. To further verify the results, LY294002 was used to inhibit the PI3K/AK signaling pathway of HCC cells. We found that the abilities of migration and invasion were decreased in MHCC-97L-TFAP4 cells when treated with LY294002 (Figure 5(b)). Western blot results also showed that the expressions of p-AKT^{Ser473}, p-GSK3β^{Ser-9}, and p-NF-κB^{P65} were markedly decreased in MHCC-97L-TFAP4 cells when treated with LY294002, subsequently leading to the increased expression of E-cadherin and the decreased expression of N-cadherin, Vimentin, and MMP-9 (Figure 5(c)). These results further demonstrated that TFAP4 promoted the invasion and metastasis of HCC by activating

Table 2: Univariate and multivariate analysis of the overall survival (OS) in HCC patients.

Variables	Univariate analysis (OS)			Multivariate analysis (OS)		
	P value	95% CI of HR	HR	*P* value	95% CI of HR	HR
Age	0.636	0.667-1.941	1.318			
Gender	0.114	0.839-5.128	2.075			
HBsAg	0.517	0.654-2.330	1.234			
AFP	0.193	0.863-2.071	1.337			
Tumor size	<0.001	1.739-4.462	2.786	0.005	1.254-3.528	2.104
Number of tumors	0.001	1.391-3.315	2.148			
Encapsulation	0.455	0.753-1.882	1.191			
Vascular invasion	0.001	1.528-4.732	2.689			
Differentiated	0.066	0.970-2.580	1.582			
Micrometastasis	0.122	0.864-3.454	1.727			
TNM	<0.001	2.187-5.768	3.551	<0.001	1.676-4.955	2.882
BCLC	<0.001	1.466-3.855	2.377			
TFAP4	<0.001	1.500-3.712	2.360	<0.001	1.705-4.314	2.712

Table 3: Univariate and multivariate analysis of the disease-free survival (DFS) in HCC patients.

Variables	Univariate analysis (DFS)			Multivariate analysis (DFS)		
	P value	95% CI of HR	HR	*P* value	95% CI of HR	HR
Age	0.731	0.584-1.445	0.918			
Gender	0.017	1.182-5.443	2.537			
HBsAg	0.240	0.810-2.317	1.370			
AFP	0.019	1.071-2.184	1.529			
Tumor size	<0.001	1.696-3.543	2.451	<0.001	1.838-3.880	2.671
Number of tumors	0.008	1.142-2.477	1.682			
Encapsulation	0.753	0.729-1.549	1.062			
Vascular invasion	0.001	1.528-4.732	2.689	<0.001	1.696-4.441	2.744
Differentiated	0.106	0.929-2.158	1.416			
Micrometastasis	0.076	0.947-2.989	1.682			
TNM	<0.001	1.732-4.093	2.663			
BCLC	0.014	1.114-2.592	1.700			
TFAP4	<0.001	1.401-2.884	2.010	<0.001	1.450-3.001	2.086

the PI3K/AKT signaling pathway. Finally, we confirmed that TFAP4 promoted invasion and metastasis by inducing EMT and regulating MMP-9 expression via activating the PI3K/AKT signaling pathway in HCC.

4. Discussion

Although there have been considerable advances in methods to diagnose and manage HCC, doctors are still not optimistic regarding the prognosis of this debilitating condition [2]. Early detection, diagnosis, and treatment are key factors that can improve the postoperative survival of HCC patients. Currently, there are few tumor markers for the diagnosis and prediction of recurrence of HCC. AFP is the most commonly used tumor marker of HCC; however, its sensitivity is only 60% [18]. Identifying novel biomarkers may assist in the formulation of novel means to enhance current diagnostic and treatment modalities of HCC. In the present study, we demonstrated that TFAP4 is highly expressed in HCC and may function as a useful prognostic biomarker in HCC patients. We also found that TFAP4 can promote the invasion and metastasis of HCC cells by activating the PI3K/AKT signaling pathway.

TFAP4 has been suggested to encode a c-MYC-inducible repressor of p21 [19], with the inhibition of p21 expression closely associated to a poorer prognosis [20]. Previous studies [12–15] have confirmed that overexpression of TFAP4 predicts poor prognosis in various malignancies, alluding to its potential usefulness as a biomarker that can predict the progression and prognosis of cancer. But in HCC, TFAP4 has rarely been studied. Recently, Song et al. [17] found that TFAP4 was highly expressed in HCC and could promote tumor formation by activating the Wnt/β-catenin pathway. However, they only analyzed a small sample size of case data from one medical center and only studied the role of TFAP4 in promoting tumor formation and proliferation of HCC

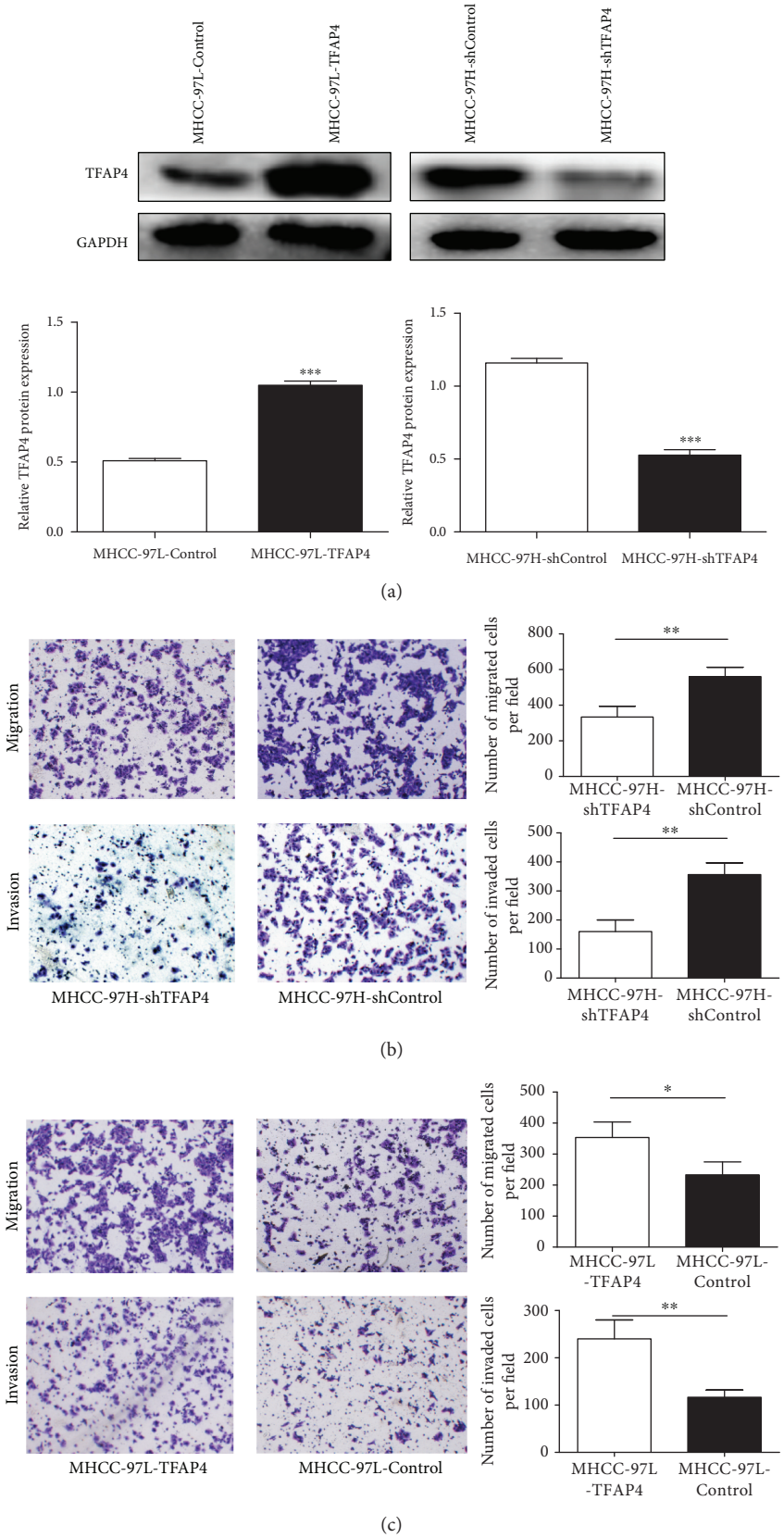

Figure 3: Effects of TFAP4 on HCC cell migration and invasion. (a) Western blot verified the transfection efficiency of TFAP4. (b) Effects of silencing TFAP4 expression on invasion and migration of HCC cells (×100). (c) Effects of overexpression of TFAP4 on invasion and migration of HCC cells (×100). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

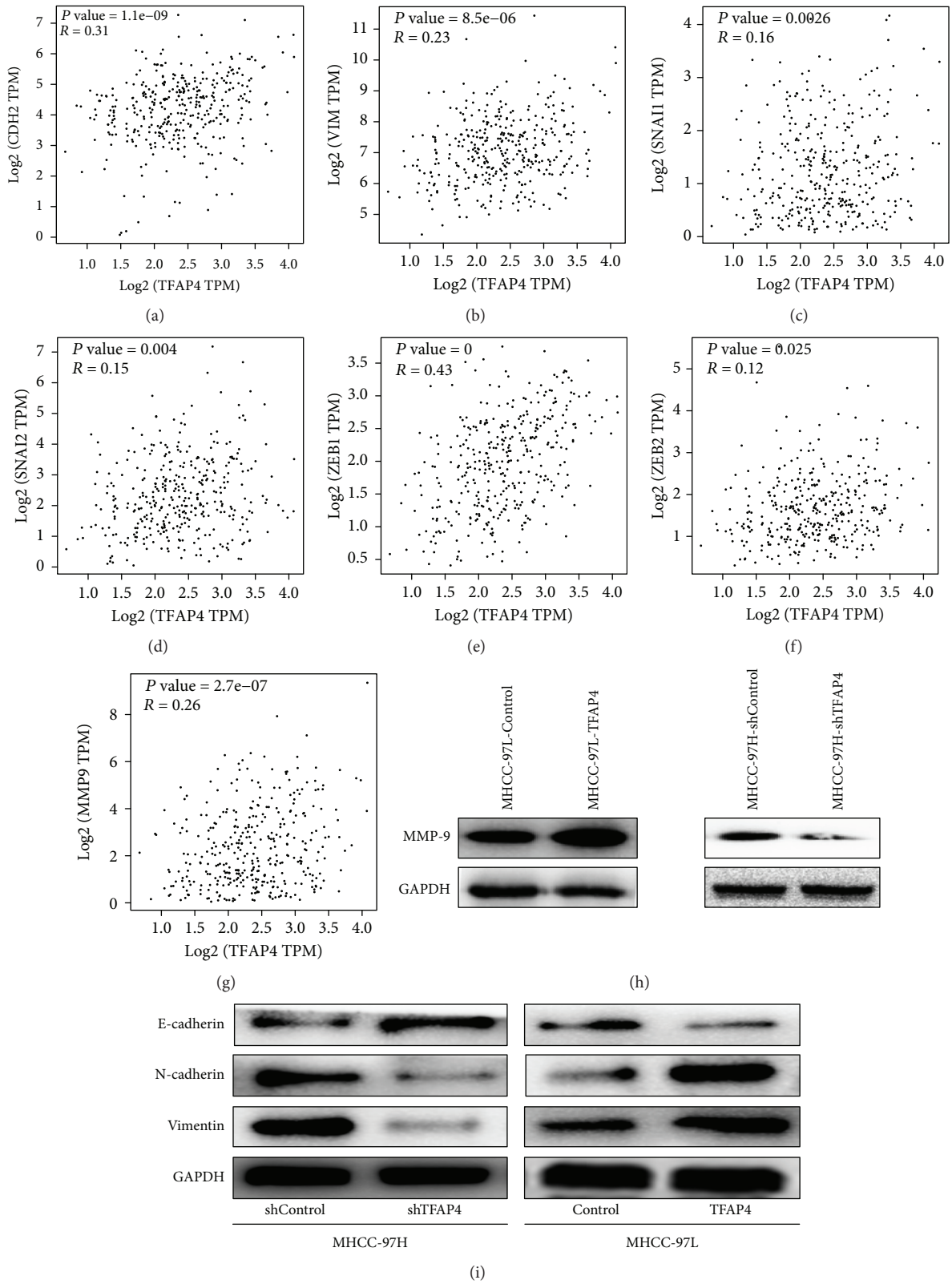

Figure 4: TFAP4 induced EMT and promoted the expression of MMP-9. (a) Correlation between TFAP4 and N-cadherin (CDH2). (b) Correlation between TFAP4 and Vimentin. (c) Correlation between TFAP4 and Snail (Snail1). (d) Correlation between TFAP4 and Slug (Snail2). (e) Correlation between TFAP4 and ZEB1. (f) Correlation between TFAP4 and ZEB2. (g) Correlation between TFAP4 and MMP-9. (h) Protein expression of MMP-9 in HCC cells of each group. (i) Protein expression of EMT markers in HCC cells of each group.

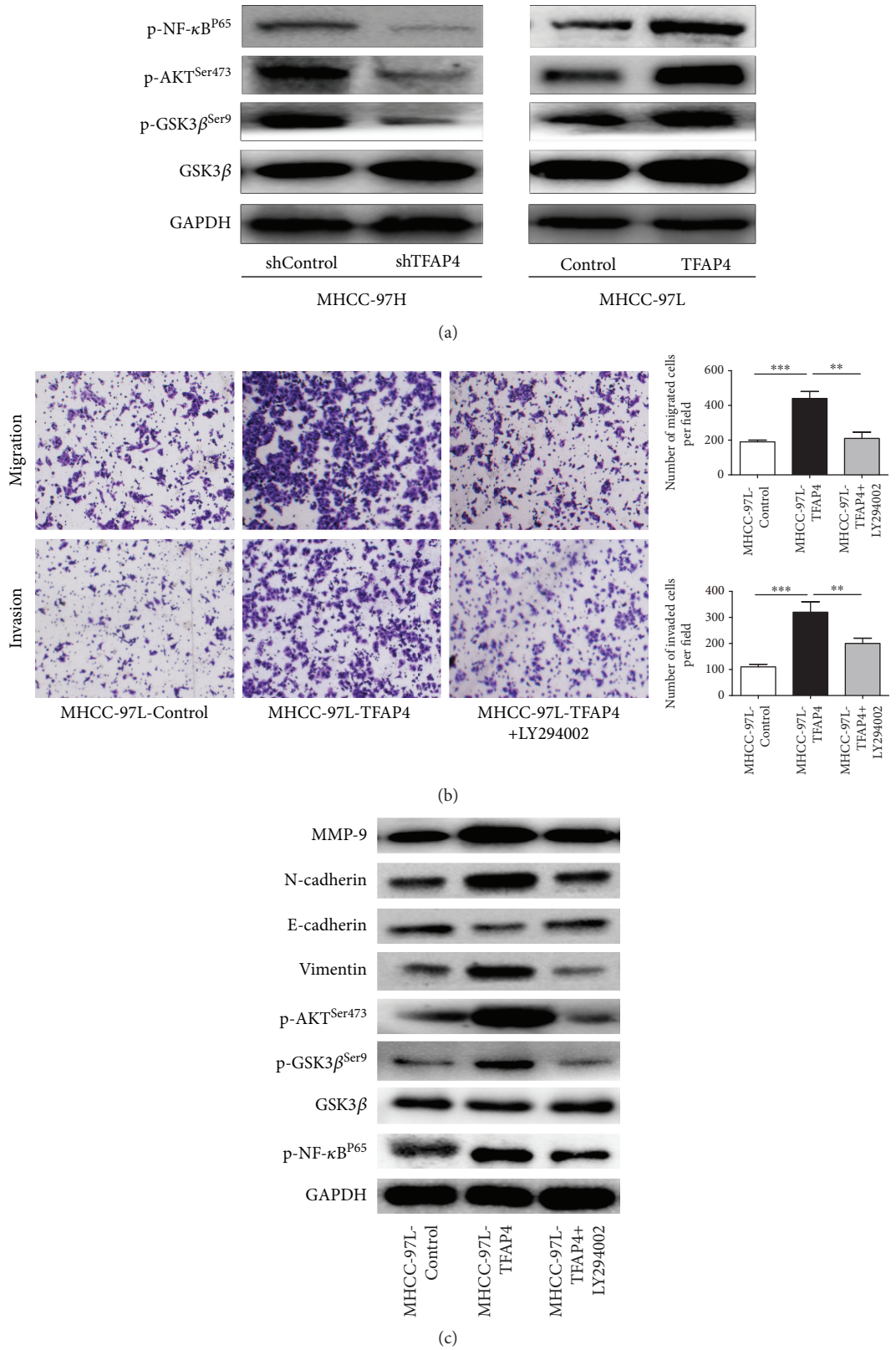

Figure 5: TFAP4 promoted the invasion and metastasis of HCC cells by activating the PI3K/AKT signaling pathway. (a) Western blot detected the protein expression of key components of the PI3K/AKT signaling pathway. (b) Effects of inhibiting the AKT signaling pathway on invasion and migration of HCC cells (×100). (c) Western blot detected the expression of each protein in HCC cells treated with LY294002. $^{**}P < 0.01$; $^{***}P < 0.001$.

cells. The effect of TFAP4 on the invasion and metastasis of HCC remains to be explored. In this study, we demonstrated an overexpression of TFAP4 in HCC tissues and cells. Furthermore, survival analysis was performed on 362 HCC patients derived from the TCGA database and 217 HCC patients enrolled from two medical centers to determine the relationship between prognosis and TFAP4 expression. Results revealed that higher TFAP4 expression conferred a significantly poorer prognosis, while subsequent Cox regression showed that TFAP4 expression was a risk factor that independently affected both OS and DFS. These results are similar to the previous research, but we also found that the expression of TFAP4 in MHCC-97H cells was higher than that in MHCC-97L cells. These two cell lines were isolated from MHCC-97 cells according to their different metastatic potentials [21]. It indicated that TFAP4 was related to the metastasis of HCC cells. These findings suggested that TFAP4 was an oncogene and functions in the tumorigenesis and metastasis of HCC.

Metastasis of tumor cells is a multistep, complex process, including exit of tumor cells from the primary tumor site, invasion of the extracellular matrix, dissemination via the blood or lymph circulation, and arrival at distant organs. Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells change into a mesenchymal phenotype, which is an important participant in the process of tumor metastasis [22]. Matrix metalloproteinases (MMPs) are proteinases that can degrade the extracellular matrix. It is generally accepted that tumor invasion and metastasis depend on the role of MMPs [23]. Since patients with high expression of TFAP4 had a higher risk of recurrence and metastasis, we further explored the role of TFAP4 in the invasion and metastasis of HCC. We successfully constructed the stable silenced and overexpressed TFAP4 cell lines. Transwell migration and invasion assay showed that TFAP4 could promote the invasion and metastasis of HCC cells. Wang et al. [24] found that TFAP4 could upregulate LAPTM4B and induce the occurrence of EMT to promote cell proliferation and migration. TFAP4 also promoted metastasis by regulating EMT in colorectal cancer [16]. Through the analysis of the TCGA database, we found that the expression of TFAP4 was positively correlated with the expression of EMT markers, related transcription factors, and MMP-9. Western blot results also confirmed that TFAP4 could promote EMT and the expression of MMP-9. We confirmed that TFAP4 regulated EMT and the expression of MMP-9 to promote the metastasis of HCC cells.

Currently, many signaling pathways have been confirmed to be involved in the regulation of HCC cell invasion and metastasis, the PI3K/AKT signaling pathway is one of the classical pathways. Previous studies have confirmed that activation of AKT signaling pathways could promote EMT in tumor cells [25, 26]. An et al. [27] found that KLF5 can induce EMT via activating PI3K/AKT/Snail signaling in HCC. Jiang et al. [28] confirmed that PRMT9 promoted HCC invasion and metastasis by activating PI3K/Akt/GSK3β/Snail signaling. Our western blot results also showed that TFAP4 could activate the AKT signaling pathway and regulate the activation of GSK3β and NF-κB. GSK3β is an important component downstream of the AKT signaling pathway, and phosphorylation of GSK3β at Ser-9 leads to its reduced activity and promotes EMT [29]. In addition, activation of the AKT pathway leads to the activation of NF-κB in HCC [30]. Previous studies have shown that NF-κB can directly bind to the promoter of MMP-9 and regulate its expression [31–33]. Our experimental results also confirmed that TFAP4 could regulate the activation of GSK3β and NF-κB by activating the PI3K/AKT signaling pathway, subsequently leading to the increased expression of MMP-9 and EMT. Furthermore, our western blot results also showed that TFAP4 could not regulate the activation of GSK3β and NF-κB after inhibiting the PI3K/AKT signaling pathway with LY294002, nor could it promote the invasion and metastasis of HCC. Taken together, we confirmed that TFAP4 could promote the invasion and metastasis of HCC by inducing EMT and promoting the expression of MMP-9 via activating PI3K/AKT signaling pathways.

In conclusion, we have showed that TFAP4 is a valuable prognostic biomarker in determining the likelihood of tumor metastasis and recurrence, as well as the long-term survival rates of HCC patients. Furthermore, we confirmed that TFAP4 could promote the invasion and metastasis of HCC via activating the PI3K/AKT signaling pathway. Therefore, TFAP4 may serve as a new biomarker of HCC with prognostic value. Exploring the regulatory mechanism of TFAP4 will also contribute to the development of new prevention and treatment strategies for HCC.

Data Availability

The data used in this study are available from the corresponding authors upon request.

Ethical Approval

This study was approved by both medical institutions' Ethics Committee.

Consent

All the patients signed the informed consent.

Conflicts of Interest

The authors report no conflicts of interest in this work.

Authors' Contributions

Tao Huang, Qi-Feng Chen, Bo-Yang Chang, and Lu-Jun Shen contributed equally to this work.

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Research Article

An Oncogenic Role for Four-Jointed Box 1 (FJX1) in Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is a highly metastatic cancer prevalent in Southern China and Southeast Asia. The current knowledge on the molecular pathogenesis of NPC is still inadequate to improve disease management. Using gene expression microarrays, we have identified the *four-jointed box 1* (*FJX1*) gene to be upregulated in primary NPC tissues relative to nonmalignant tissues. An orthologue of human *FJX1*, the *four-jointed (fj)* gene in Drosophila and *Fjx1* in mouse, has reported to be associated with cancer progression pathways. However, the exact function of FJX1 in human is not well characterized. The overexpression of FJX1 mRNA was validated in primary NPC tissue samples, and the level of FJX1 protein was significantly higher in a subset of NPC tissues (42%) compared to the normal epithelium, where no expression of FJX1 was observed ($p = 0.01$). FJX1 is also found to be overexpressed in microarray datasets and TCGA datasets of other cancers including head and neck cancer, colorectal, and ovarian cancer. Both siRNA knockdown and overexpression experiments in NPC cell lines showed that FJX1 promotes cell proliferation, anchorage-dependent growth, and cellular invasion. Cyclin D1 and E1 mRNA levels were increased following FJX1 expression indicating that FJX1 enhances proliferation by regulating key proteins governing the cell cycle. Our data suggest that the overexpression of FJX1 contributes to a more aggressive phenotype of NPC cells and further investigations into FJX1 as a potential therapeutic target for NPC are warranted. The evaluation of FJX1 as an immunotherapy target for NPC and other cancers is currently ongoing.

1. Introduction

Nasopharyngeal cancer (NPC) is a highly metastatic cancer that is particularly prevalent in Southeast Asia and Southern China with an incidence rate of 20-50 per 100,000 persons per year [1, 2]. According to GLOBOCAN 2018, a total of 129,097 new cases of NPC and 72,987 death cases worldwide is estimated in 2018 [3]. Radiotherapy is effective against early-stage NPC; however, over 70% of cases present with late-stage disease and only 10-40% of these patients survive for more than 5 years [4, 5]. Currently, the mainstay treatment for locoregional advanced cases of NPC is concurrent chemoradiotherapy. Unfortunately, undesirable complications such as xerostomia, cranial nerve neuropathy, and osteoradionecrosis occur frequently after the treatment because of the location of the tumour at the base of the skull that is closely surrounded by and in close proximity to many vital structures such as the brain, spinal cord, eyes, ear, and parotid glands that result in high morbidity and poor quality of life [6, 7].

NPC is consistently associated with Epstein-Barr virus (EBV) infection [8], and it is well recognized that EBV alters many functional properties that are involved in tumour progression [9]. However, the exact contribution of EBV to

the pathogenesis of NPC is not fully understood. The molecular events that drive the progression of NPC are still elusive, and it is likely that a better understanding of its molecular pathogenesis will lead to the identification of novel biomarkers and therapeutic targets.

High-throughput analyses such as microarrays and genome sequencing have facilitated the discovery of many potential biomarkers for diagnostics, therapeutics, and prediction of treatment outcome. Using platforms such as microarrays, whole genome sequencing, targeted deep sequencing, and SNP arrays, several important pathways such as ErbB-PI3K, Akt-mTOR, Notch, and NF-κB were found to be frequently altered among NPC patients [10–12], suggesting that these pathways could be targeted for treating NPC.

Using expression microarrays, we have previously identified genes that are differentially expressed in EBV-positive primary NPC tumours compared to cancer-free nasopharyngeal tissue samples [13]. From this study, several potential oncogenes or candidate biomarkers such as FJX1, WNT5A, CLDN1, FGFR3, FZD6, RALA, and CLCA2 were shortlisted based on previous reports on their involvement in cancer pathophysiology, neoplasia, and embryogenesis process. Among these genes, the human four-jointed box 1 (FJX1) was chosen to be further characterized by its function in NPC development. In this study, we show that FJX1 is overexpressed at both the mRNA and protein levels in a subset of primary NPC tumours, as well as in other cancers. Following the knockdown of the gene in FJX1-overexpressed cells and overexpression of FJX1 in low-expressing cell lines, respectively, we demonstrated that this gene promotes the proliferation, anchorage-independent growth, and invasion of NPC cells. Together, our data suggest a possible role for FJX1 in the pathogenesis of NPC.

2. Materials and Methods

2.1. Cell Lines and Tissue Biopsies. The cell lines used in this study included HK1 and HeLa/T, a HeLa hybrid that was previously reported as a cell line derived from NPC (TW04). Cell line authentication showed that the STR profile of this line shared 80.0% similarity with D98-AH2, a clone of HeLa (Supplementary Table 1). The cell lines were cultured in RPMI-1640 (Gibco, USA), supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA) and 10% fetal bovine serum (FBS) (Gibco, USA). Fresh-frozen nasopharyngeal biopsies ($n = 16$) from newly diagnosed and treatment-naïve patients from Tung Shin Hospital (Kuala Lumpur, Malaysia) were included in the quantitative real-time PCR analysis (qRT-PCR). Clinical information on these biopsies indicated that 14 were undifferentiated Epstein–Barr virus-encoded small RNA- (EBER-) positive NPC, while 2 were EBER-negative nasopharyngeal biopsies with no evidence of malignancy. A further 43 paraffin-embedded archival tissue samples from NPC patients diagnosed at Tung Shin Hospital, Kuala Lumpur, were obtained and included in our analysis. As controls, 11 archived tissue samples of the nonmalignant nasopharynx tissues with no evidence of NPC were obtained from the Tung Shin Hospital, Kuala Lumpur, and Nilai Cancer Centre, Negeri Sembilan (Supplementary Table 2). Ethical approval for this study was obtained from the independent ethics committee of respective institutions, and written informed consent was obtained from all patients before tissue collection.

2.2. Quantitative Real-Time PCR (qRT-PCR). Transcriptomic levels of target antigens after knockdown assays were determined by qRT-PCR. Briefly, total RNA from fresh frozen biopsies was extracted using NucleoSpin® RNA II (Macherey-Nagel, Germany), and 1 μg total RNA was subjected to reverse transcription using SuperScript II reverse transcriptase (Invitrogen, USA). The resulting cDNA was used as input to perform qRT-PCR to detect FJX1 and CCND1 expression at the transcription level using corresponding TaqMan probes (FJX1: Hs00534909_s1; CCND1: Hs00765553_m1; Applied Biosystems, US) and primer pairs (FJX1 5′-CCCGCAAAGGTGTCTAAAAACT-3′ and 5′GTGCTGGCACAGTAAAGAATCCT 3′; CCND1 5′-CCCTGACGGCCGAGAAG-3′ and 5′-GGTCTGCGCGT GTTTGC 3′). The transcriptional level of CCNE1 was assessed using SYBR Green (Life Technologies, US) with primer 5′-CTGGATGTTGACTGCCTTGAATT-3′ and 5′-GCGACGCCCCTGAAGTG-3′. The ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) was used for both the TaqMan and SYBR Green assays. In parallel, GAPDH (5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′) was included to serve as an internal control for normalization, and all experiments were performed in triplicate following the manufacturer's protocol. A no-template control was included to assess the overall specificity of the reaction. The nonmalignant nasopharynx tissue with the highest level of FJX1 was used to compare with FJX1 expression of NPC tissues. Fold changes in gene expression between nonmalignant nasopharynx tissue and tumour samples were measured using the comparative threshold cycle method ($\Delta\Delta$Ct), as described previously [13]. Compared to the controls, expression of the target genes was considered to be significantly upregulated when the fold change of expression > 2 with the $p < 0.05$ by a two-tailed t-test.

2.3. Immunohistochemistry. Immunohistochemistry (IHC) was performed to determine the expression levels and localization of FJX1 protein using Dako REAL™ EnVision Detection System (DakoCytomation, Denmark). Antigen retrieval was performed by microwaving the sections in high pH antigen retrieval solution (Link Technology, UK) for 30 minutes, and endogenous peroxidase activity was quenched using Dako REAL™ Peroxidase-Blocking Solution for 15 minutes. Tissue sections were washed with phosphate-buffered saline (PBS) and then incubated with anti-human FJX1 rabbit polyclonal antibody at 1 : 500 (Aviva Systems Biology, USA). After 1 hour of incubation at room temperature, the sections were washed once in Tris-buffered saline (TBS) buffer containing 0.1% Tween-20 (TBST) for 5 minutes, and this was further incubated with the HRP-

conjugated polymer for 30 minutes. After washing for 5 minutes in TBST, the expression of FJX1 was visualized with the development of diaminobenzidine (DAB) as a chromogen, and the sections were counterstained with Mayer's haematoxylin (BDH Laboratories, UK), washed in tap water, dehydrated in graded alcohols, cleared in xylene, and mounted in DPX (Fluka BioChemika, USA). Immunoreactivity of epithelial cells (cancer and normal) was scored based on a 4-point intensity scoring system: 0 = negative expression, 1 = weak positive, 2 = moderate positive, and 3 = strong positive. The ductal epithelium of the NPC tissues which were always stained positive served as the internal positive control for IHC.

2.4. Knockdown and Overexpression of FJX1. To investigate if FJX1 drives the progression of NPC, HK1 which expresses a high level of FJX1 endogenously was selected in the knockdown assay, while HeLa/T cells with low levels of FJX1 were chosen in the overexpression study. To knockdown the expression of FJX1 expression in NPC cell line HK1, ON-TARGETplus SMARTpool siRNA targeting FJX1 (HK1-siFJX1) or Non-Targeting Pool siRNA (HK1-siNT) was used at 50 nM (Dharmacon, USA). Approximately, 7.5×10^4 cells per well were seeded into 6-well plates, cultured overnight, and transfected with the relevant siRNA using DharmaFECT 1 transfection reagent (Dharmacon, USA). The FJX1 siRNA pool contained 4 sequences targeting different coding regions of FJX1, and the sequences include 5′-CGGAGCAGAUUCAGGGCGA-3′, 5′-AGUACAAUG GACCGACUUA-3′, 5′-UCGACUACCUGACGGCCA-3′, and 5′-GGACUUAGUGUCACCGGGA-3′. The effect of FJX1 knockdown was confirmed by qRT-PCR analysis as a good anti-FJX1 antibody is not available.

To overexpress FJX1 in the HeLa/T cells, firstly, the coding region of FJX1 was amplified from cDNA of NP460 cells by PCR using the GC-RICH PCR System (Roche, Germany) and PCR products after purification were cloned into the pcDNA3.1-V5/His-B vector using BamH1 and EcoR1 restriction sites. Approximately, 3×10^4 HeLa/T cells per well were seeded into 6-well plates, cultured overnight, and then transfected with the plasmids using Lipofectamine™ 2000 transfection reagent (Invitrogen, USA). Cell lines overexpressing FJX1 are referred to as HeLa/T-FJX1, while cell lines transfected with vector control were referred to as HeLa/T-vector.

2.5. Western Blotting. To confirm the expression of FJX1 after transfection, transfected HeLa/T cells were lysed on ice in RIPA buffer (50 mM Tris pH8, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, 150 mM NaCl) containing the protease inhibitor cocktail (Roche, USA). Cell lysates were collected after centrifugation at 14000 rpm for 15 minutes at 4°C, and the concentration of the total cellular protein for each sample was determined using the Bradford protein assay (Pierce Biotechnology, USA). Extracted proteins (50 μg) were denatured at 95°C for 5 minutes followed by a 12% SDS-polyacrylamide gel separation, and separated proteins were transferred onto the Immobilon-P membrane (Millipore, MA, USA). Then, blots were blocked with 5% skimmed milk in TBST for 1 hour and further incubated with the indicated primary antibodies (anti-V5: 1 : 2000; Abcam, UK; anti-α-tubulin: 1: 2000; Sigma-Aldrich, USA) overnight at 4°C. The next day, blots were washed (3 times for 5 minutes each) in TBST buffer and subsequently incubated with the relevant IRDye Odyssey secondary antibodies (LI-COR Biosciences, USA) for 1 hour. After washing (TBST), detection was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). Due to the unavailability of a good antibody against FJX1, an anti-V5 antibody was used to detect FJX1 overexpression after transfection.

2.6. Cell Proliferation Assay. To determine the role of FJX1 in promoting cell proliferation, HeLa/T-FJX1 and HeLa/T-vector or HK1-siFJX1 and HK1-siNT were seeded at 5×10^4 cells per well in 6-well plates in triplicate, 24 hours post-transfection. The cells were trypsinized everyday for 5 days and counted on a CASY cell counter (Roche Innovatis AG, Germany). The mean cell counts and standard errors were calculated.

2.7. Soft-Agar Colony Formation Assay. To determine the ability of FJX1 overexpression to drive cellular anchorage-independent growth *in vitro*, low-melting agarose VII (Sigma-Aldrich, USA) was used in the colony formation assays. Approximately, 24 hours post-transfection with plasmids, single-cell suspensions of 2×10^3 cells per well were plated into 6-well plates in 2 mL of RPMI containing 10% FBS and 0.5% agar on a layer of 2 mL of the same medium containing 1% agar. After 3 weeks, colonies were counted microscopically. The mean colony counted from each field and standard errors were calculated.

2.8. In Vitro Invasion Assay. Invasion assays were carried out using Matrigel-coated 8 μm pore size polycarbonate Transwell filters (Corning, USA) as described previously [14]. Approximately 24 hours post-transfection with siRNAs targeting FJX1 or FJX1 expression plasmids, cells were treated with 15 μg/mL mitomycin C (Sigma-Aldrich, USA) for two hours to inhibit proliferation. Approximately 5×10^4 cells in 200 μL migration buffer (0.1% BSA/serum-free RPMI) were plated into the upper chamber and left to incubate for 48 hours. Total cells having invaded to the lower chamber were trypsinized and counted using the CASY cell counter (Roche Innovatis AG, Germany).

2.9. Bioinformatic Analysis. Microarray data from the Gene Expression Omnibus (GEO) repository [15] and the datasets that compared gene expression levels in NPC and colorectal and ovarian tumours to normal tissues from the respective sites were analysed. For the RNA-Seq data, transcriptomic data from 43 head and neck cancer tissue samples and matched normal samples from TCGA database [16] were analysed.

2.10. Statistical Analysis. For the immunohistochemical analysis, the comparison between groups was determined by Fisher's exact test. For *in vitro* studies, statistical differences between experimental groups were evaluated by two-tailed t-test and $p < 0.05$ was taken as being statistically significant.

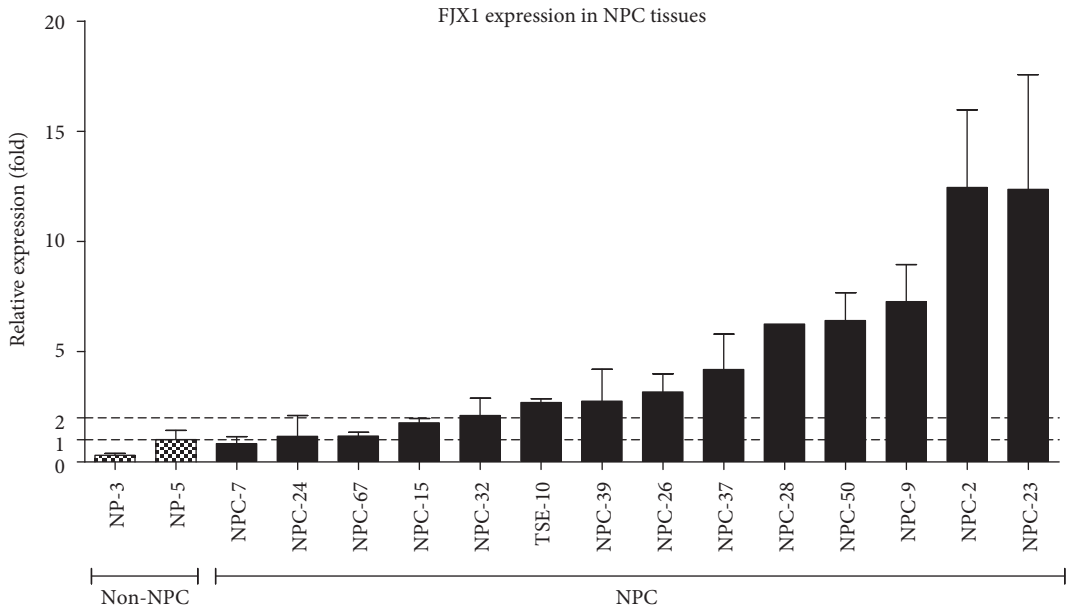


Figure 1: Overexpression of FJX1 in NPC. Quantitative PCR showed that when compared with two biopsies of the normal nasopharynx (NP-3 & NP-5), FJX1 significantly elevated at transcriptomic levels in 10 of the 14 samples that showed significant upregulation by array analysis.

3. Results

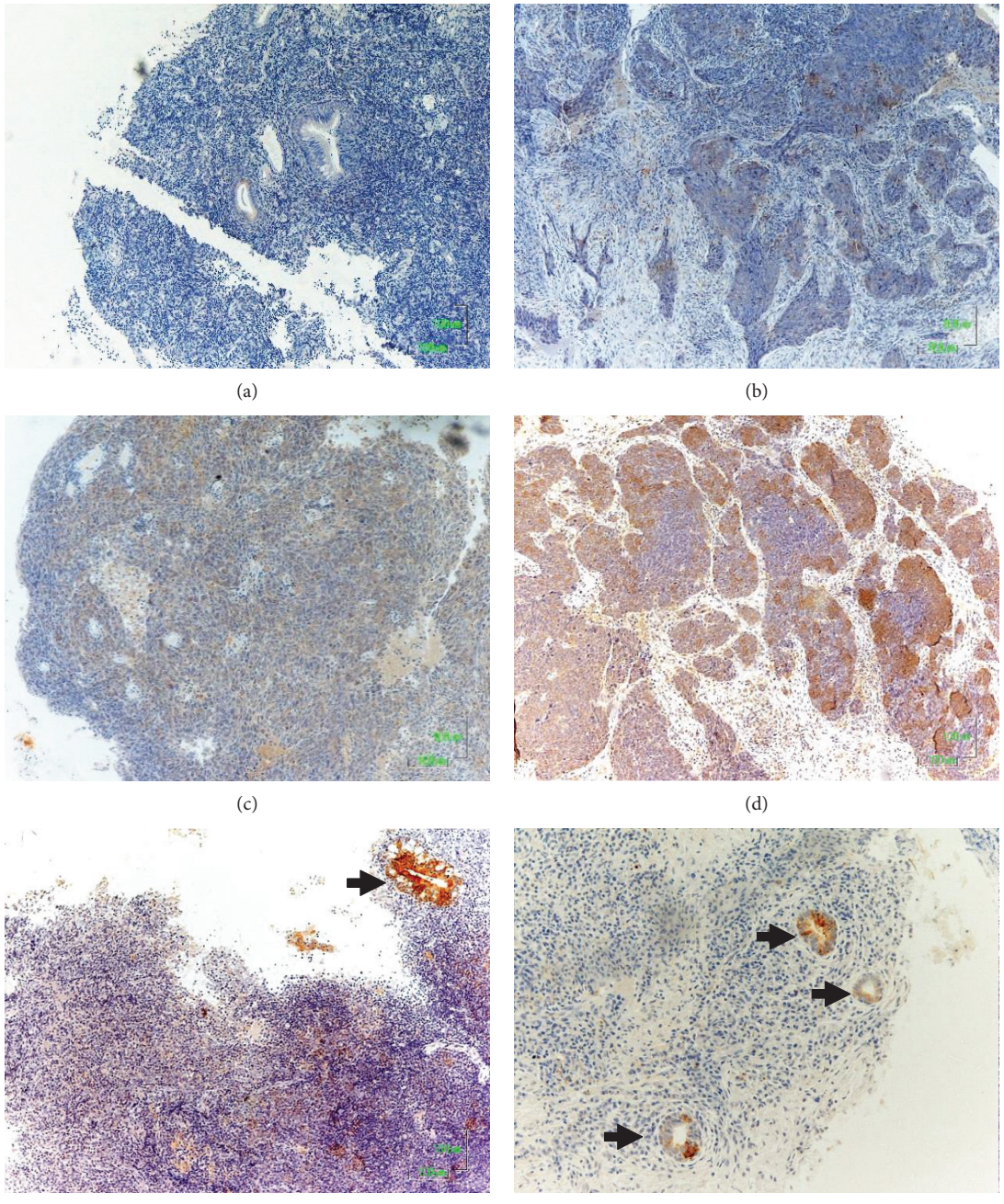
3.1. FJX1 Is Overexpressed in NPC. We first sought to confirm that FJX1 is overexpressed in NPC, having previously shown that FJX1 was significantly upregulated in 14/25 (56%) cases by microarray analysis (Supplementary Figure S1a) [13]. Fourteen samples that were from the previous microarray analysis were available for qRT-PCR analysis, and 10/14 demonstrated at least a 2-fold increase in FJX1 levels when compared with the two nonmalignant controls (Figure 1).

The expression and cellular localization of FJX1 protein were further examined in an independent set of 43 formalin-fixed paraffin-embedded primary NPC and 11 nonmalignant nasopharyngeal samples by immunohistochemistry (Figure 2). The normal epithelium in the control samples and the normal epithelium adjacent to carcinoma were consistently negative for the expression of FJX1 in all 11 nonmalignant samples (Figure 2(a)). FJX1 expression was detected in 18 of 43 (42%) of the tumours examined, consistent with our microarray and qRT-PCR observations that FJX1 is upregulated in NPC. The expression of FJX1 was cytoplasmic, and the intensity ranged from weak to strong (Figures 2(b)–2(d)). The ductal epithelium of the nasopharynx tissues served as an internal positive control and consistently stained positive for FJX1 (Figures 2(e) and 2(f)). There was no significant association between the expression of the FJX1 protein and the clinical parameters (T, N, and overall stage) (Supplementary Table 3).

As a dysregulated gene in one cancer is typically also dysregulated in other types of cancer [17], we sought to explore whether this is also the case for FJX1. Indeed, besides our own microarray dataset (GSE13597), we found that FJX1 is also overexpressed in independent NPC microarray dataset (GSE12452) (2.8 fold), as well as colorectal and ovarian cancers [18, 19] (2.4- and 2.3-fold, respectively; $p < 0.01$) when compared to normal tissues (Table 1). Notably, RNA-Seq data from 43 head and neck cancers with matched normals from the TCGA database [16] consistently showed that tumour samples had a 2-fold elevated level of FJX1 when compared to the matched normals ($p < 0.01$) (Supplementary Figure S1b).

3.2. FJX1 Promotes NPC Cell Proliferation through Cell Cycle Regulation. To investigate the phenotypic impact of FJX1 *in vitro*, HK1 cells which express high levels of FJX1 were transfected with FJX1 siRNAs. Two days following siRNA transfection, FJX1 levels were reduced by ~60% while the expression of GAPDH remained unaffected, indicating the specificity of the FJX1 siRNA (Figures 3(a) and 3(b)). On the other hand, a HeLa hybrid NPC cell line, HeLa/T, which expresses low levels of FJX1 was transfected with pcDNA3.1-V5/FJX1 and the expression confirmed by Western blotting. Three FJX1-specific bands of different molecular weights at around 50 kDa were clearly detected in the Western blotting analysis using a V5 antibody, potentially reflecting differential posttranslational modifications (Supplementary Figure S2). Previous study showed that two conserved potential N-linked glycosylation sites in the mouse are also present in human FJX1 [20]. A similar observation and multiple glycosylation sites in human FJX1 protein were also observed by Al-Greene et al. [18]. The occurrence of multiple bands on Western blotting could be due to the existence of an unglycosylated form as well as two other different forms of glycosylation at single or multiple sites.

Next, proliferation assays were performed on these two lines over 5 days. For HK1 cells with reduced FJX1 protein levels, the growth was consistently slower when compared to the HK1 cells transfected with siNT ($p = 0.03$). In contrast,



(a) (b) (c) (d) (e) (f)

Figure 2: Immunohistochemistry confirms the upregulation of FJX1 protein expression in NPC, but not in nonmalignant nasopharynx tissues. Representative images of (a) nonmalignant tissues and (b)–(d) NPC samples with a staining intensity of 1-3 are shown. (e) and (f) Endothelial ductal served as an internal positive control for the staining.

Table 1: Microarray datasets that reported the overexpression of FJX1.

GEO reference	Type of cancer	Tumour sample	Normal sample	Fold change	p value
GSE13597	NPC	25	3	1.89	0.04
GSE12452	NPC	31	10	2.82	$8.73E-07$
GSE32323	Colorectal	17	17	2.44	$2.49E-06$
GSE14407	Ovarian	12	12	2.23	0.000629

HeLa/T with elevated levels of FJX1 grew significantly faster ($p = 0.01$), when compared with their respective control cells (Figures 3(c) and 3(d)). The data suggest that high FJX1 levels promote cell proliferation. To examine whether FJX1 enhances cell proliferation through cell cycle regulation, the expression of two molecules crucial to cell cycle progression, cyclin D1, and cyclin E1, were investigated. mRNA levels of cyclin D1 and E1 were reduced by approximately half following the knockdown of FJX1 in HK1 cells but were 5-fold elevated in FJX1-overexpressing HeLa/T cells (Figures 3(e)

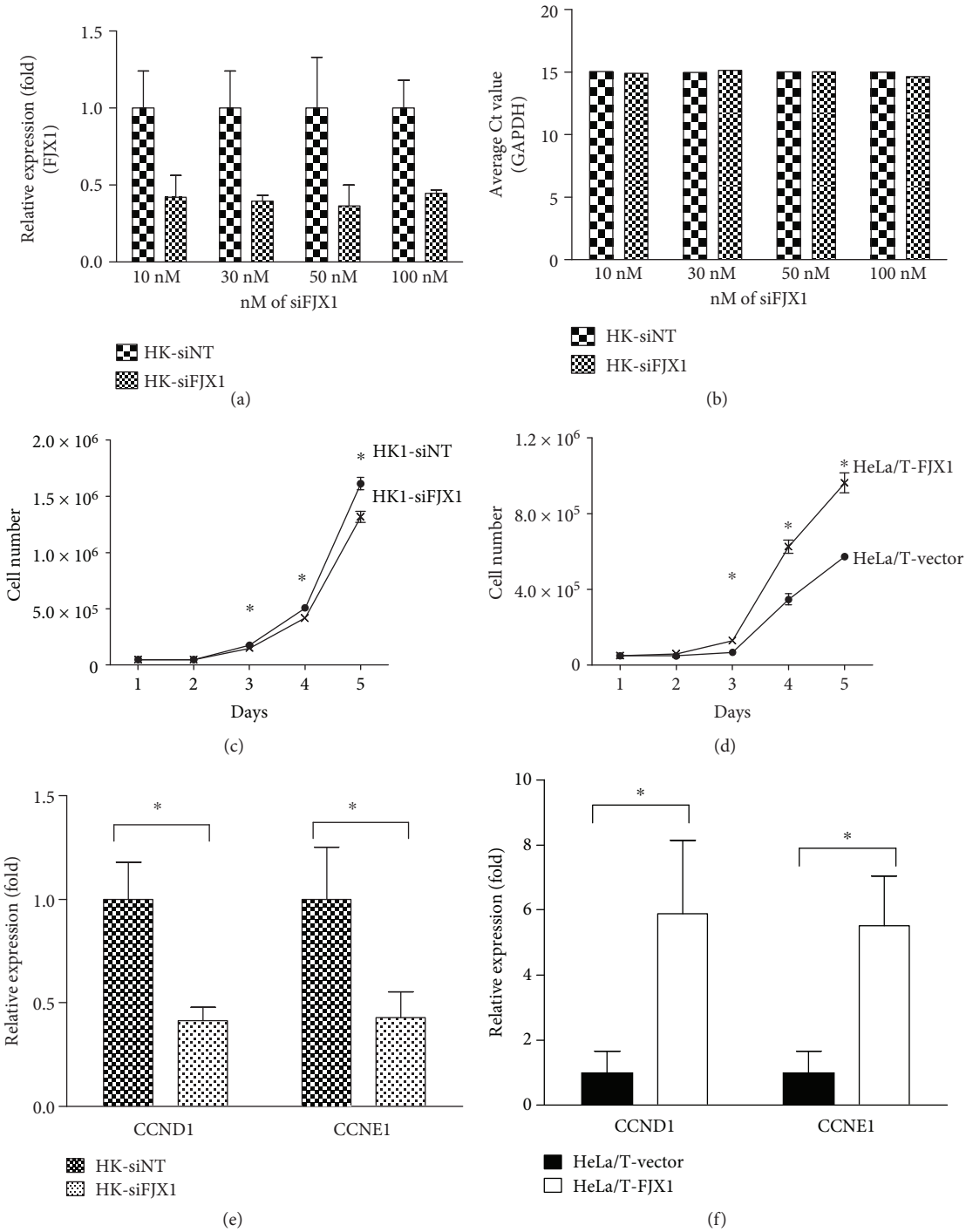


Figure 3: FJX1 promotes cell proliferation. (a) HK1 cells transfected with different concentrations of nontargeting (siNT) or FJX1-specific (siFJX1) siRNA were analysed by qRT-PCR. (b) Transfected HK1 cells with various concentrations of siFJX1 do not affect the expression of the housekeeping gene GAPDH. (c) HK1 cells transfected with FJX1 siRNA grew significantly slower than control cells, (d) while overexpression of FJX1 in HeLa/T cells resulted in significantly increased cell proliferation. The mRNA levels of cyclin D1 and cyclin E1 were determined in (e) HK1 and (f) HeLa/T cells by qRT-PCR. Reduced levels of cyclin D1 and E1 were evident following the knockdown of FJX1 in HK1 cells, whereas HeLa/T cells expressing FJX1 showed higher levels of these two cyclins compared to the vector control cells. The expression levels of the controls were normalized to 1 ($*$ denotes $p < 0.05$).

and 3(f)). These data suggest that FJX1 could promote cell growth by increasing the expression of positive regulators of cell cycle progression.

3.3. FJX1 Enhances Anchorage-Independent Growth. Soft agar colony formation assays were performed to determine the contribution of FJX1 to anchorage-independent cell

growth, one of the most reliable markers of malignant transformation [21]. Comparing HeLa/T cells overexpressing FJX1 to those transfected with vector alone, we demonstrated that a high expression of FJX1 resulted in a significant increase in the number of colonies formed ($p = 0.04$; Figure 4), suggesting that FJX1 can contribute to a more aggressive phenotype in cancer. This assay was not performed on HK1 cells as these cells inherently do not form colonies in soft agar.

3.4. FJX1 Promotes NPC Cell Invasion. To examine the role of FJX1 in cell invasion, *in vitro* Matrigel Transwell assays were performed. As shown in Figure 5, 24 hours post-transfection with siFJX1 and siNT, the invasive ability of HK1 cells was reduced by ~35% following FJX1 knockdown ($p = 0.02$). Similarly, HeLa/T-FJX1 cells were also significantly more invasive than the vector control cells ($p = 0.01$). The capability of FJX1-expressing cells to invade through the Transwell membrane suggests that FJX1 enhances the invasiveness of tumour cells and could contribute to the ability of tumour cells to metastasize.

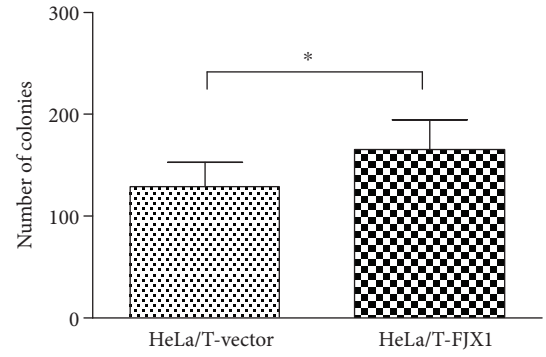


Figure 4: FJX1 enhances anchorage-independent growth. Compared to the control cells, the overexpression of FJX1 in HeLa/T cell lines resulted in significantly increased numbers of colonies (∗ denotes $p < 0.05$).

4. Discussion

In this study, we identified FJX1 to be overexpressed in NPC, and exogenous expression of FJX1 conferred a survival advantage to cancer cells. While much remains unknown about the function of FJX1, emerging studies have identified FJX1 to be amplified or overexpressed in several types of cancers including breast [22, 23], lung [24], ovarian [19], colorectal [18], and head and neck cancers [25, 26], suggesting that it could be a genetic driver for cancer. FJX1 is also found to be overexpressed in endometrium tissues from women with endometriosis [27]. Although endometriosis is considered a nonmalignant condition, the characteristics of endometriosis such as infiltration of endometriosis tissues to surrounding organs and presence of angiogenesis resembles a tumour-like behavior to some extent [18, 28, 29]. Microarray studies including ours demonstrated that *FJX1* is overexpressed in NPC relative to noncancerous tissues [13, 30, 31]. Overexpression of *FJX1* in the transcriptomic level was also observed in RNA-Seq data from 43 head and neck cancers compared to matched normal samples from the TCGA database. Further, *FJX1* amplification was also detected in 16/16 oral cancer cell lines derived from oral squamous cell carcinoma when compared to normal keratinocytes [32]. Taken together, these independent datasets demonstrate that FJX1 is consistently upregulated in cancers, particularly in head and neck cancer, underscoring the rationale of studying the function of this gene in these cancers.

Embryogenic development and cancer progression shared many similar characteristics. For example, during the developmental process, cells within the embryo go through rapid proliferation, placental implantation (invasion), cellular movement (migration), and formation of blood vessels (angiogenesis) [33, 34]. Further, genes and pathways which are tightly regulated in embryonic development are often found to be dysregulated or reactivated in cancers [35, 36]. Studies have shown the involvement and importance of four-jointed (Fj) protein in the developmental processes of various organisms. In *Drosophila*, Fj (the Drosophila equivalent of FJX1) is required for proximodistal growth and regulation of planar polarity where Fj mutant flies have shorter wings and legs compared with those that are wild-type [37, 38], while in crickets, Fj is involved in the regeneration of limbs by restoring the growth of amputated legs to normal size [39]. Similar observations were also reported in chick embryo development, where the chick *four-jointed* (*fjx*) expression was detected in limb buds at different phases and functions by first controlling the limb outgrowth then tissue differentiation at a later stage [40]. In mouse, Fjx1 is found in the developing brain and homozygous Fjx1 mutant mice showed abnormal morphology of dendrite arbors in the hippocampus of the brain. When cultured *in vitro*, hippocampal neurons from these mutant mice showed a higher level of dendrite extension and branching [41]. These studies underscore the importance of FJX1 in the development of different organisms. Although the exact function of FJX1 in human is not fully understood, data from the developmental studies suggests that the function of FJX1 in cancer development could be linked to its role in promoting growth and cell movement.

The human FJX1 protein shares 29% and 88% identity with its orthologue in *Drosophila* and mouse, respectively [42]. Fjx1 is a transmembrane kinase which is also known as the Golgi kinase four-jointed, Fj. Several studies on the orthologue of FJX1, the Fj, or Fjx1 in *Drosophila* and mouse Fjx1 revealed that Fjx1 is Notch-induced and plays a role in several important pathways including the Fat/Hippo pathway. Signaling pathways such as the Hippo, Wnt, Hedgehog, and Notch pathways were reported to be dysregulated and confer to the progression of many cancers [41, 43]. These pathways have already been shown to be important for head and neck cancers [16, 44]. Our study has revealed that the expression of FJX1 has a significant correlation of cancer cell proliferation and enhances anchorage-independent growth. A recent study showed that FJX1 is one of the downstream effectors of the frequently altered Hippo signaling pathway and FJX1 is a direct target of the YAP and TAZ oncogenes within this pathway [45].

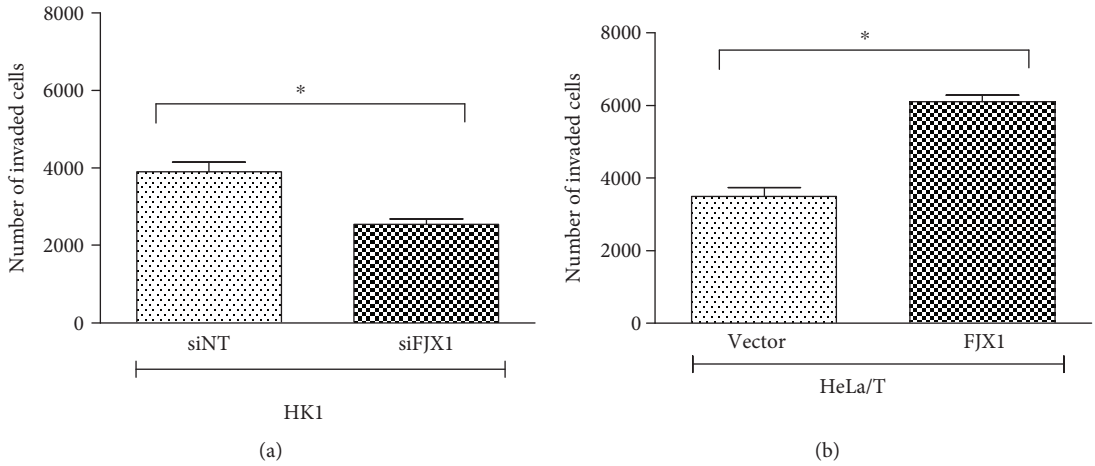


Figure 5: FJX1 promotes cell invasion. Inhibition of FJX1 in HK1 cells resulted in a 35% reduction in the number of cells invading compared to controls. By contrast, FJX1-expressing HeLa/T cells were significantly more invasive than the vector control cells ($*$ denotes $p < 0.05$).

Functionally, the Hippo pathway plays a critical role in regulating cell proliferation and apoptosis [46–48]. For the first time, we demonstrate that FJX1 plays a role in controlling cell proliferation and this is associated with an upregulation of cyclins including cyclin D1 and E1. Of note, Fjx1 has also been shown to form a feedback loop where overexpression of the protein further regulates the activation of YAP [49, 50].

In this study, we also demonstrated that FJX1 could increase the invasive potential of cancer cells. This phenotype has been shown *in vivo* in a non-small cell lung cancer model where Fjx1 overexpression was found to be associated with increased metastasis [51]. FJX1 could potentiate invasion perhaps through its involvement in regulating planar cell polarity (PCP), a process where cells orientate themselves within the tissue plane for collective cell movement [52]. While PCP is a critical process in wound repair and development, facilitating processes such as gastrulation, neural tube closure, and heart morphogenesis, to name a few, dysregulation could lead to pathological conditions including cancer invasion and metastasis. PCP is coordinated by Fat and Dachsous where these proteins are proposed to physically interact in a heterotypic fashion to mediate PCP [53]. Fj regulates the phosphorylation state of the cadherin repeats of Fat and Ds affecting their ability to interact with one another [37, 53–55] which could explain why upregulation of FJX1 results in increase invasion in head and neck cancer cells.

5. Conclusions

In summary, we demonstrated that FJX1 confers a survival advantage and invasive potential to nasopharyngeal carcinoma and is a likely target for the treatment of cancer. Its involvement in multiple important pathways controlling tumour growth and cell movement rationalises the development of targeted therapies against this protein. An *in vitro* study on the development of immunotherapy using peptides derived from FJX1 as a treatment for head and neck cancer including NPC has shown the ability of these peptides to elicit patients' T cell cytotoxic response towards tumour cells expressing FJX1 [56, 57]. The efficacy and safety of FJX1 peptide vaccine are currently under investigation using an *in vivo* mouse model.

Data Availability

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

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Materials

Table S1: STR profile of TW04 with a match of ≥80.0% to HeLa D98-AH2 clone. Table S2: histological characteristics of nonmalignant nasopharyngeal tissue samples. Table S3: correlation between FJX1 expression and clinicopathological characteristics of NPC tissue samples. Figure S1: transcriptomic levels of FJX1 in cancer. (a) Previous microarray

analysis (GSE13597) showed that the mRNA level of the FJX1 transcript is increased in NPC biopsies and NPC cell lines compared to normal nasopharynx tissue, suggesting FJX1 as a potential biomarker for NPC. (b) FJX1 mRNA levels also reported being elevated in head and neck cancer patients when comparing RNA-Seq data of 43 cancer tissues with matched normal samples from the TCGA database. Figure S2: FJX1:V5 overexpression in HeLa/T cell was confirmed using the V5 antibody. Three bands with different molecular weights of FJX1 protein were detected in Western blotting. *(Supplementary Materials)*

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Research Article

DNA Methylation Profiles and Their Diagnostic Utility in BC

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Biomarkers, including DNA methylation, have shown a great potential for use in personalized medicine for BC and especially for the diagnosis of BC in developing countries. According to the bisulfite sequencing PCR in twelve specimens (BC and matched normal tissues), nine genetic probes were designed to detect the frequency of methylation of the promoters in a total of 302 paired cases of BC and matched normal breast tissues. Finally, a total of 900 serum samples were used to validate the use of these methylation biomarkers for clinical diagnosis of BC. A high frequency of promoter methylation of *SFN*, *HOXA11*, *P16*, *RARβ*, *PCDHGB7*, *hMLH1*, *WNT5a*, *HOXD13*, and *RASSF1a* was observed in BC tissues. The methylation frequencies of *HOXD13* and *hMLH1* increased with the progression of BC. The methylation frequencies of *HOXD13* and *WNT5a* were significantly higher in BC. We found that methylation modification-positive samples were most consistently associated with luminal BC. Finally, we confirmed that *RASSF1a*, *P16*, and *PCDHGB7* displayed a significant sensitivity and specificity as diagnostic biomarkers for BC ($P < 0.001$), and a panel that combined these three genes displayed increased significance (AUC, 0.781; $P < 0.001$). These data suggest that epigenetic markers in serum can potentially be used to diagnose BC. The identification of additional BC-specific methylated genes would improve the sensitivity and specificity of this approach. This study could also indicate that different molecular subtypes of BC are caused by distinct genetic and epigenetic mechanisms.

1. Introduction

Breast cancer (BC) is a complex and heterogeneous disease and a leading cause of death among women. Some regional surveys have indicated that the incidence of BC is also rising in Chinese women [1]. Approximately, the incidence of BC was 26.86% and the mortality of BC was 6.95% among Chinese women in 2015. High incidence is concentrated between 45 and 59 years old [2].

Carcinogenesis is a multistep process that results from the accumulation of genetic and epigenetic alterations [3]. Recent reviews have emphasized that epigenetic abnormalities might play an influential role in the earliest steps of cancer initiation and the progression of malignancies [4, 5], especially because the methylation of a normal allele can serve as a "second hit" that leads to gene inactivation when paired with mutations in the opposite allele [6]. Approximately 40-50% of human genes have CpG islands (CGIs) located in or near the promoter and/or first exon, and the methylation of these CGIs is critical to regulate the expression of these genes [7]. Alterations in the methylation status of DNA are among the most frequent molecular changes that are associated with human cancers [8].

In BC, many studies have investigated methylation patterns as potential biomarkers for detection, subtype classification, risk stratification, monitoring prognoses, and predicting susceptibility or responsiveness to a particular therapy [9, 10]. However, in spite of the promise of such biomarkers, several barriers continue to prevent rapid progress toward using these markers in clinical applications. Major limitations to the further development of these markers in clinical applications might be that many studies have focused on investigating the methylation patterns in circulating free DNA (cfDNA) derived from the serum of healthy women

and women with BC, but studies rarely use benign breast tissues as the control to identify the potential clinical applications of using serum DNA methylation as a biomarker. In addition, these studies have investigated fewer BC and matched control specimens, and validation with larger patient cohorts has not been pursued [11, 12]. Other limitations include the utilization of different technologies by different laboratories, resulting in a range of detection sensitivities, a varying emphasis on quantitation, and the utilization of different sample processing methodologies and different reference materials as controls during analysis of hypermethylation degrees when using the same technology [13]. We therefore investigated a new diagnostic tool for BC and sought to overcome these barriers by using methylation genes as cancer biomarkers. We determined whether these genes could also be useful markers for predicting a prognosis in BC patients according to the progression of the cancer. These were *ductal carcinoma in situ* (DCIS), *invasive ductal carcinoma* (IDC), and *invasive ductal carcinoma plus lymph metastasis* (IDC-L).

In the present study, twelve candidate markers for BC (*SFN* (*14-3-3σ*), *HOXA11*, ARID1a, CBX7, DLC1, *P16*, *RARβ*, *PCDHGB7*, *hMLH1*, *WNT5a*, *HOXD13*, and *RASSF1a*) were studied with regard to their detection in BC tissues and matched serum samples. These genes have previously been shown to undergo cancer-specific methylation in breast tissues in the TCGA database [14] and other reports of clinical or fundamental studies [15–20]. These markers are representatives of a variety of cellular pathways, including DNA binding, cell cycle/checkpoint control, developmental regulation, chromatin binding, cell adherence, and cytokine activity. In addition, *HOXA11*, *HOXD13*, and *PCDHGB7* were confirmed as early methylated genes when human mammary epithelial cells (HMEC) converted into cancer cells in our previous study and in other studies [19]. We examined the methylation status of the promoters of these candidate genes in two independent sets (test and validation) using a total of 302 paired tissue/normal samples. A matched serum detection assay of the validation set ($n = 194$) was then used to confirm the results obtained for the top hypermethylated genes from both the test and the validation sets, to show the reliable cfDNA methylation markers that diagnose BC. Finally, we identified the methylation biomarkers that best differentiated BC in a total of 900 serum samples that included samples from 300 BC patients, 300 patients with benign breast diseases, and 300 healthy women.

2. Materials and Methods

2.1. Patients, Sample Collection, and DNA Extraction. All individuals signed surgical or clinical research consent forms allowing tissue and serum collection in accordance with the regulations approved by the IRB Committee of Harbin Medical University. This research was completed in compliance with the Helsinki Declaration. A brief outline of the study process is shown in Supplemental Figure 1. The study extended over biomarker development phases 1 and 2, which were based on Early Detection Research Network (ERDN) guidelines [13, 21]. All the tissue and serum samples were obtained from patients and healthy persons undergoing physical examination at the Affiliated Tumor Hospital of Harbin Medical University, Harbin, China, from 2014 to 2017. Fresh-frozen specimens derived from cancerous and self-pair normal breast tissues (≥5 cm distant from the tumor tissue) were obtained from patients who underwent a mastectomy for BC. The benign breast diseases included fibroadenoma, benign phyllodes tumors, mastopathy, papilloma, duct ectasia, and hamartoma (Supplemental Table 3). Healthy serum samples were acquired from the Affiliated Tumor Prevention and Treatment Institution of Harbin Medical University. All H&E slides were reviewed by two independent pathologists to determine the integrity of the tumor specimen (tumor content of >70%) and the normal tissue blocks, in which no tumor cells were observed.

All samples were classified as one of four types of primary BC lesions: (1) pure DCIS, 100 cases; (2) IDC, 100 cases; and (3) IDC-L, 102 cases. Genomic DNA was isolated from fresh-frozen primary breast tumors and matched normal breast tissues. Samples were pretreated with proteinase K (20 mg/mL) at 55°C overnight and DNA was then extracted using an AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., CA, USA). Approximately 5 mL of peripheral blood was drawn into a blood collection tube prior to a physical examination or surgery, and all samples were transferred to the study laboratory within 4 hours of collection for processing. Circulating free DNA (cfDNA) was obtained from 1 mL of serum using a QIAamp Circulating Nucleic Acid Kit [22] (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Immunohistochemistry and Molecular Subtypes. The monoclonal ER antibody was obtained from Ventana (catalog no. 760-2596). The monoclonal PR antibody was obtained from Dako (catalog no. M3569). Nuclear labeling for ER positivity or PR positivity was required in greater than 1% of cells [23]. HER-2 IHC was performed using the Dako HercepTest kit according to the manufacturer's protocol. Cases were scored using the established criteria as 0, 1+, 2+, or 3+. Fluorescence in situ hybridization analysis to determine Her-2 amplification was performed on all 2+ (equivocal) cases using the PathVysion kit (Des Plaines, IL). To qualify as Her-2 positive in this study, a case had to demonstrate either a 3+ IHC score or a Her-2 fluorescence in situ hybridization amplification ratio of greater than 2.2. Cases were categorized into one of four categories based upon accepted and previously validated IHC surrogate profiles of BC. Luminal A tumors were immunoreactive for ER and/or PR and negative for Her-2 or low proliferation. Tissue that was ER+ and/or PR+, either Her2+ and/or highly proliferative, was considered luminal B tumors. The Her-2 subtype was defined as ER-, PR-, and Her2+. Basal-like tumor was the most controversial type. On the basis of the published criteria, basal-like cases were defined as tissues with a triple-negative phenotype (ER-/PR-/Her2-). We therefore used triple-negative BC (TNBC) instead.

IHC for p53 (Ventana, monoclonal antibody, catalog no. 760-2542) and Ki-67 (Ventana, monoclonal antibody,

catalog no. M7240) only showed nuclear labeling. For p53, a labeling score indicating that >30% of the nuclei were labeled was defined as aberrant overexpression (which correlates well but not perfectly with the presence of p53 mutation) [24]. The Ki67 cut-off point was 20%, and this was used to designate a tumor as highly proliferative when assigning samples to subtype groups [25].

2.3. Bisulfite Treatment, Sequencing, and MethyLight. Bisulfite conversion of genomic DNA was performed using an EZ DNA Methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Converted DNA was amplified using PCR as described in Supplemental Excel 1. For each BSP, ten positive clones were sequenced in both directions by the Life Technologies Lab (Invitrogen, Burlington, ON, CA).

According to the results of BSP sequencing, we selected the probable promoter CpG islands that contained the methylated variant sites to design probes for each gene (Supplemental Figure 1). A detailed list of the nucleotide sequences corresponding to the MethyLight primers and probes in the promoter or 5′ end region of all analyzed loci is provided in Supplemental Excel 2. TaqMan MGB (Applied Biosystems, Foster City, CA, USA) PCR was performed using primers specific for the bisulfite-converted methylated sequence of a particular locus. *Globin* reference primers were used separately. The TaqMan MGB probes showed a significant improvement in assay specificity, and their smaller size allowed for a more flexible assay design.

MethyLight is highly specific, sensitive, and reproducible. It can also rapidly detect biologically relevant information in patient samples. MethyLight is a PCR-based method that requires only very small amounts of DNA of modest quality, and this makes it compatible with small biopsies and paraffin-embedded tissues [26]. MethyLight could therefore be a utility tool for use in clinical applications [13]. The majority of studies that have used percentage of methylated reference (PMR) as a method for evaluating methylation have reported positive results. But the cut-off value for PMR varies when used with MethyLight in different studies [15, 27–29]. This is likely the result of not using self-matched normal tissue as a control in studies that instead use *SssI*-treated human peripheral white blood cell DNA from the same person or from healthy people as the control. This comparison may not accurately reflect positive methylation cases, because methylation modification is influenced by many factors, including lifestyle, environmental exposure, ethnicity, age, and tissue heterogeneity [26, 30]. In this study, we compared BC tissue to matched normal breast tissue (distant from tumor mass ≥ 5 cm) from the same person, and the percentage of samples that were methylated at a specific locus was statistically calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (CT_{\text{Target gene}} - CT_{\text{Reference}})$ sample $- (CT_{\text{Target gene}} - CT_{\text{Reference}})$ control (matched normal tissue from the same patient) [31]. All samples were assayed in duplicate, and to validate the results of the $2^{-\Delta\Delta Ct}$ method, the amplification efficiencies of the test genes and a reference gene, Globin, were examined using serial dilutions of DNA over a 100-fold range and using gene-specific primers for each gene and Globin. The ΔCt ($CT_{\text{Target gene}} - CT_{\text{Reference}}$) was calculated for each DNA dilution, and a plot of the log DNA dilution vs. ΔCt was constructed. A cut-off value of ≥1.5 [32, 33] (allelic gene methylation) was determined to indicate a positive result. The analysis of cfDNA methylation frequency was also performed using the MethyLight method. We used $2^{-\Delta Ct}$ (ΔCt was calculated as $CT_{\text{Target gene}} - CT_{\text{Reference}}$) in a ROC curve analysis to determine both sensitivity and specificity in comparison of results between BC and control samples (including healthy women and patients with benign breast diseases).

2.4. Statistical Analysis. Data were analyzed using Student's *t*-tests, Fisher's exact tests, Kruskal-Wallis *H*, ROC curve analyses, and Mann-Whitney *U* tests. All tests were performed using SPSS 17.0. A P value of <0.05 was considered significant.

3. Results

3.1. Prescreening of the Promoter CpG Islands of Candidate Genes to Select Methylation Targets Using BSP. From the TCGA database, gene methylation biomarkers identified for the diagnosis of other tumors in the previous studies, and the distinct methylation genes identified during the conversion from human normal mammary epithelial cells to BC cells, we selected *SFN* (14-3-3σ), *HOXA11*, ARID1a, CBX7, DLC1, *P16*, *RARβ*, *PCDHGB7*, *hMLH1*, *WNT5a*, *HOXD13*, and *RASSF1a*, which represent a variety of different pathways that are involved in cancer (Supplemental Excel 3). Initially, we evaluated the CpG islands of all of these genes [34], and the highly dense regions containing the CpG sites in the CpG islands were sequenced using BSP in six paired cases of BC tissues and matched normal breast tissues (Supplemental Figure 2). DLC1 was eliminated because the highly dense CpG region could not be amplified using BSP. We then selected variant methylation sites that were methylated in at least half of the BC tissues and unmethylated in the matched breast normal tissues to design the probes for MethyLight (Supplemental Table 1).

3.2. Determination of Methylation Frequency in an Appropriate Gene Evaluation Set of Patients. Next, all residual tissue specimens were divided into two data sets: the test set (108 paired cases of BC tissues and matched normal breast tissues) and the validation set (194 paired cancer tissues and matched normal tissues). All of these samples were obtained from BC patients aged 40-60 years old to rule out the effect of age on DNA methylation. The other clinicopathological factors, including the pathological type, histological grade, BMI, and tumor size, were not different ($P > 0.05$, Supplemental Table 2) between the test set and the validation set.

We investigated the methylation frequency of eleven genes between BC tissues and matched normal breast tissues in the test set using MethyLight. Significantly high methylation frequencies were detected for nine genes in BC tissues from the test set. Moreover, we next confirmed these results

using the same nine methylation probes in the validation set, and we found that they also displayed the high methylation frequencies (Supplemental Table 4). In conclusion, a total of nine methylated genes, including *SFN*, *HOXA11*, *P16*, *RARβ*, *PCDHGB7*, *hMLH1*, *WNT5a*, *HOXD13*, and *RASSF1a*, were methylated with significantly higher frequency in BC tissues than in matched normal breast tissues from 302 BC patients. The average methylation frequencies for all of the genes in the BC tissue group are shown in Supplemental Table 5. Among all of these markers, *PCDHGB7* was most often methylated (78.81%), whereas the lowest methylation frequency was observed for *WNT5a* (28.48%).

3.3. Methylation Frequency during the Progression of BC. We categorized all of the malignant samples into four groups according to the histopathology of BC, including DCIS, IDC, and IDC-L. The results of the methylation frequency analysis for all nine genes is illustrated in Table 1. All of the genes displayed widespread aberrant promoter CpG island methylation. The frequency of *HOXD13* and *hMLH1* methylation significantly increased with the progression of the disease from in situ to invasive cancer ($P < 0.001$ and $P < 0.05$, Figure 1), but there was no significant difference between IDC and IDC-L.

3.4. Methylation Profiles Associated with Molecular Subtypes and Clinicopathological Features. In each group (DCIS, IDC, and IDC-L), we classified three subgroups according to the coexistence of methylation between genes: coexistence of one to three methylation genes, four to six methylation genes, or seven to nine methylation genes (Supplemental Table 6). We found that the category with the fewest genes was the group indicating the coexistence of seven to nine methylated genes in the DCIS group (5%). To exclude contingency and on the basis of the prior studies [35], we determined that samples in which at least three genes were simultaneously methylated were likely to be affected by epigenetic modifications (especially DNA methylation modifications) and we named these "methylation modification-positive samples." Next, we analyzed the specimens that clustered with the BC molecular subtypes in different groups (Figure 2). We found that methylation modification-positive samples were consistently the luminal type of BC (Figure 3) in the DCIS, IDC, and IDC-L groups.

3.5. Evaluation of the Consistency of Methylation Frequency between BC Tissues and Matched Serum and Determination of the Best-Performing Methylation Probes in BC Diagnoses. Based on the above data, we have shown that gene methylation frequencies are significantly higher in BC tissues. Next, we considered whether methylation can be used as a diagnostic marker of BC. We used specific probes to assess the methylation of nine genes in matched serum samples in the validation set using MethyLight. The majority of the genes that were methylated in the BC tissue were also methylated in the matched cfDNA obtained from serum (in the gene methylation-positive tissues). The same genes displayed higher average frequencies in the matched serum, including *PCDHGB7*, *P16*, and *RASSF1a* (Table 2). Meanwhile, the frequency of *HOXA11* or *WNT5a* methylation was low in serum, even though the frequency of methylation of these markers in the matched BC tissues was high. In addition, *HOXD13* was only methylated in 1 cfDNA sample, and *RARβ* was only methylated in 3 samples of cfDNA. The methylation frequency of *hMLH1* was 33.33% in serum and 35.57% in tissues. Finally, the observation that frequencies increased along with the progression of BC, as observed in tissues and illustrated in Figure 3, did not recur in the serum methylation study.

Table 1: Methylation frequencies for the nine genes in breast cancer patients.

Methylated gene	Breast cancer tissue (*302 cases*) methylation frequency (%)		
	DCIS	IDC	IDC_L
SFN	*25.67*	*27.35*	*36.67*
HOXA11	*38.23*	*40.67*	*45.34*
P16	*37.45*	*44.5*	*42.11*
RASSF1a	*64.45*	*54.38*	*68.45*
PCDHGB7	*75.56*	*83.45*	*76.45*
hMLH1	**23.55**	**40.57**	**47.43**
Wnt5a	**31.34**	**34.56**	**36.45**
HOXD13	**32.34**	**65.33**	**66.78**
RARβ	*43.45*	*24.78*	*34.68*

According to the frequent study of methylation genes in breast cancer tissues and serum samples, we selected genes that had a higher methylation frequency in both the breast cancer tissues and the matched serum samples to explore the clinical utility of using such methylation biomarkers to diagnose breast cancer. *PCDHGB7*, *P16*, and *RASSF1a* (although the methylation frequency of *RASSF1a* was lower in the serum in this study, it was generally high in breast cancer tissues) were selected. We used an expanded set of serum samples that included 300 breast cancer samples, 300 samples from age-matched healthy controls, and 300 samples from age-matched patients with benign breast diseases. In the ROC curves corresponding to the three analyzed genes (Figure 4(a)), *RASSF1a* showed a sensitivity of 75%, a specificity of 62.5%, and an area under the curve (AUC) of 0.682 (95% CI, 0.645 to 0.719, $P < 0.001$). The sensitivity and specificity of *P16* were 75% and 64.33%, respectively, and the AUC was 0.687 (95% CI, 0.650 to 0.724, $P < 0.001$). *PCDHGB7* showed the highest sensitivity (84.33%). This is in accordance with our results showing that this marker showed the highest methylation frequency in breast cancer tissues (Supplemental Table 5). However, the specificity of serum *PCDHGB7* was not very high (60.33%), and the AUC for this marker was 0.660 (95% CI, 0.630 to 0.678, $P < 0.001$). Next, we performed an ROC curve analysis for a three-gene panel to determine its sensitivity and specificity for diagnosing breast cancer (Figure 4(b)). According to this analysis, the three-gene panel discriminated between breast cancer patients and controls with a sensitivity of 82.67% and a specificity of 77.83% (AUC, 0.781; 95% CI, 0.757 to 0.796, $P < 0.001$). This combination of three

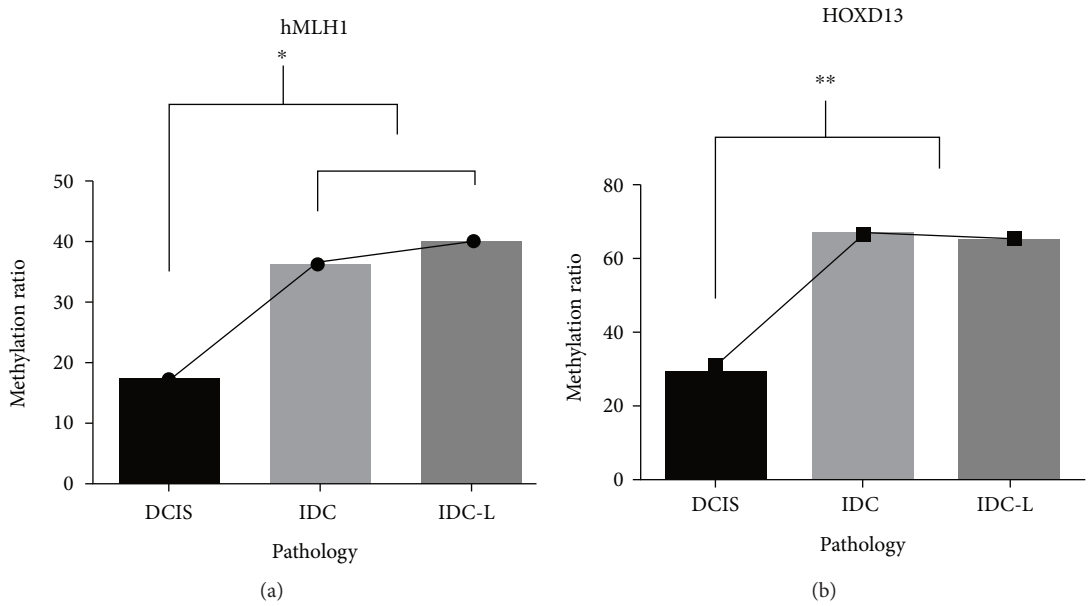

Figure 1

different methylation markers maximized their significance in the clinical diagnosis of breast cancer.

4. Discussion

The recent report has indicated that the incidence of BC in developing countries is stably increasing [35]. However, distinctive features related to BC in Asian women relative to women of predominantly European ancestry include Asian women who have larger tumors and a more advanced tumor stage at diagnosis, and these characters are associated with delayed diagnosis or more aggressive disease [1, 22, 36]. Approximately 75% of cases in Asian women are diagnosed at late and untreatable stages (clinical stages III and IV) [37, 38]. This may be due to lack of awareness, limited healthcare infrastructure, inadequate manpower, and the uneven distribution of resources. Mammography has been the "gold standard" for BC detection for decades, and it is commonly applied in western countries. However, it is not suitable for use in developing countries as a diagnostic tool for BC because of limitations, including age, the density of breast tissues, socioeconomic factors, and medical resources [34, 39]. It is therefore necessary to explore efficacious, economical, convenient, and practical diagnostic methods that are suitable for use in developing countries.

In this study, we screened the methylation status of nine genes belonging to different molecular pathways in different pathological BC and matched normal tissues. This could raise the accuracy of such biomarkers for determining a diagnosis or prognosis in BC [40, 41]. Although *RASSF1a*, *RARβ*, *SFN*, *hMLH1*, and *P16* have been widely detected in different studies [18, 30, 42, 43] and in different people [27, 44, 45], we report important data regarding the frequency of the methylation of genes in BC. We found that *HOXA11*, *PCDHGB7*, and *HOXD13* are also highly methylated in BC tissues, which was rarely reported in the prior studies, especially in DCIS. Furthermore, we show that not all of the genes that are methylated in tumor tissues are also highly methylated in serum cfDNA, as the case for *HOXD13* and *HOXA11*. Although knowledge of the underlying mechanisms involved in determining the levels of these genes in circulating DNA is still limited [46], some evidence suggests that cfDNA is released from tumors as a glyconucleoprotein complex, which might protect it from degradation by nucleases [47]. It remains unclear whether the release of tumor DNA into serum is associated with tumor necrosis, apoptotic cell death, or other selective cellular processes. Because it is presumably shed from the original primary tumor, cfDNA might be fragmented and the quantity of cfDNA is greatly reduced. It has therefore been suggested that the clinical utility of using methylated biomarkers to diagnose BC must be confirmed in the serum and not just in BC tissues. For diagnostic biomarkers of BC, we selected *RASSF1a*, which is widely used as a methylation biomarker for diagnosing BC in western countries, and *P16* and *PCDHGB7* because they are highly methylated in both BC tissues and serum. *RASSF1a* and *P16* displayed a significant utility for diagnosing BC, as found in the prior studies. However, the sensitivity and specificity observed in this study were different from that found in the previous studies [16, 48, 49]. The most important reason for this discrepancy could be that we added benign controls in this study, and this may have reduced the sensitivity and specificity of these markers in contrast with results that compare only BC and healthy samples. Other reasons could be differences in the race of the sample population or their environment, difference in methodology, and differences in the targets being investigated. Hence, in future research aimed at investigating methylation to determine diagnostic biomarkers in BC, we strongly suggest that samples should contain matched samples from patients with benign diseases. The methylation frequency observed in *PCDHGB7* may be the first time that methylation has been detected in BC tissue

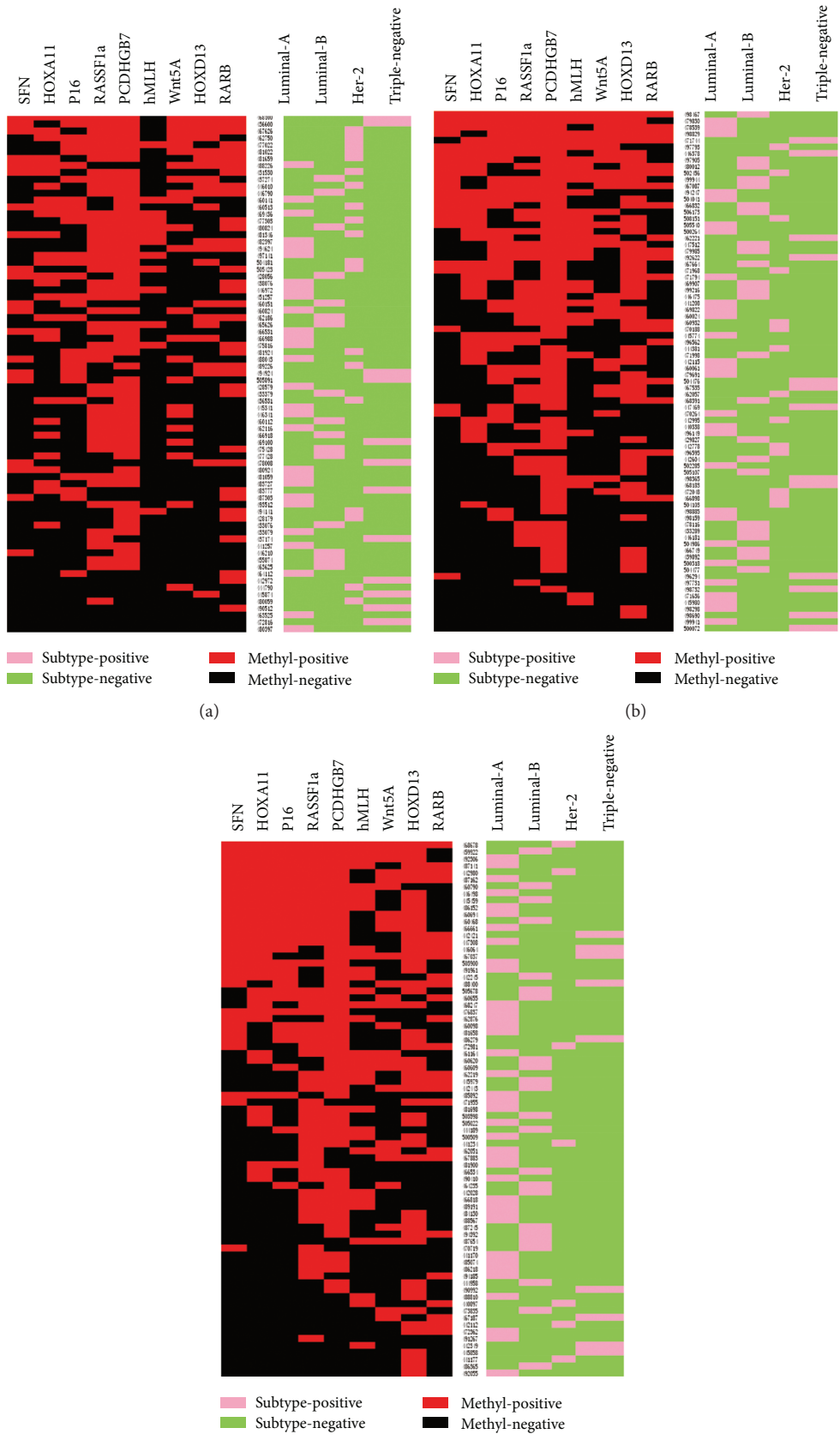
SFN
HOXA11
P16
RASSF1a
PCDHGB7
hMLH
Wnt5A
HOXD13
RARB
Luminal-A
Luminal-B
Her-2
Triple-negative
Subtype-positive
Subtype-negative
Methyl-positive
Methyl-negative
(a)
(b)
(c)

Figure 2

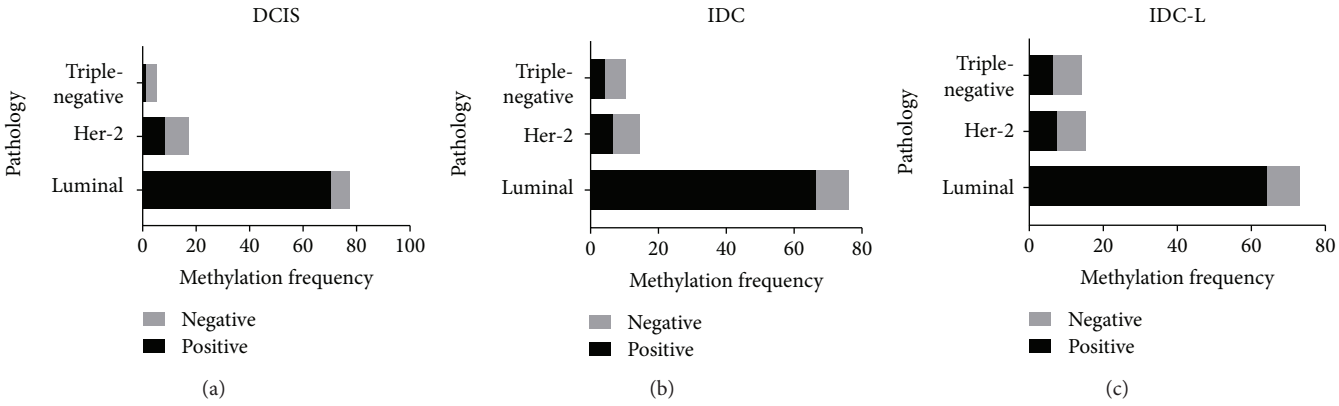

Figure 3

Table 2: Methylation frequencies of the same genes in the matched serum and methylation-positive BC tissues.

Methylated gene	Breast cancer tissue (validation test, 194 cases)		Methylation frequency (%)	Matched serum		Methylation frequency (%)
	Methyl case	Unmethyl case		Methyl case	Unmethyl case	
SFN	*61*	*133*	*31.44*	*35*	*26*	**57.38**
HOXA11	*88*	*106*	**45.36**	*15*	*73*	*17.01*
P16	*86*	*108*	**44.33**	*48*	*38*	**55.81**
RASSF1a	*117*	*77*	**60.31**	*43*	*74*	**36.75**
PCDHGB7	*156*	*38*	**80.41**	*79*	*77*	**50.64**
hMLH1	*69*	*125*	*35.57*	*23*	*46*	**33.33**
Wnt5a	*57*	*137*	*29.38*	*12*	*45*	*21.05*
HOXD13	*90*	*104*	**46.39**	*1*	*89*	*1.11*
RARβ	*71*	*123*	*36.6*	*3*	*68*	*4.23*

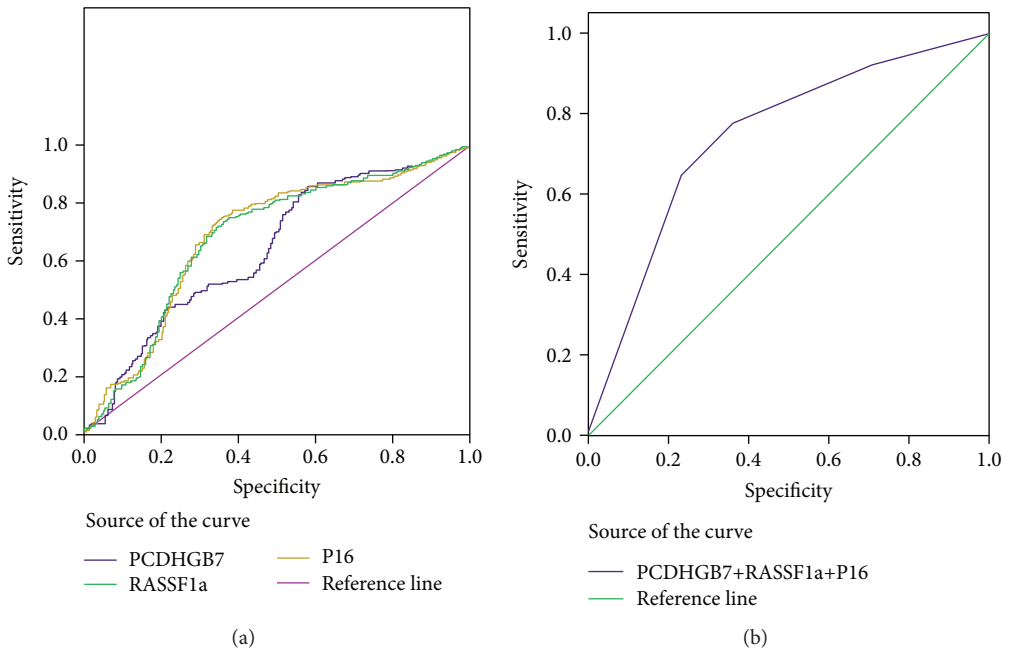

Figure 4

and serum and found to be an effective methylation biomarker for the diagnosis of BC. In addition, we also investigated the mRNA expression of *PCDHGB7* in BC tissues to confirm its methylation in BC. The results showed that *PCDHGB7* was expressed at low levels in most BC tissues (approximately 80%, Supplemental Figure 3). Finally, we confirmed that a panel of methylated biomarkers that included these three genes showed the best sensitivity and specificity for the diagnosis of BC. Therefore, in future studies, we will add more effective methylation markers to increase the sensitivity and specificity of this panel for use in diagnosing BC.

Many studies have reported that the frequency of methylation should significantly increase in parallel with the progression of cancer [19, 33] and that methylation could therefore potentially be used as a predictor during the determination of a prognosis in BC. In our study, we showed that *HOXD13* and *hMLH1*, in BC tissue methylation detections, demonstrate this phenomenon. In particular, methylation of *HOXD13*, a member of the HOX family, significantly increased in parallel with the progression of BC (from in situ to metastasis). This example was also just reported by Gupta et al. [50] in *Nature*. However, this phenomenon did not reappear for either *HOXD13* or *hMLH1*in the analysis of methylation in serum. As we discussed above, this reminds us that accordance in methylation profiles between tissues and the matched serum samples is not perfect, as reported by Korshunova et al. [51]. Many methylation biomarkers that have been detected in BC tissues in the prior reports may not be suitable for clinical diagnoses of BC unless they are also analyzed in the serum. We also found that the methylation frequency of *RARβ* showed a significant variation: 43.45% in DCIS, 24.78% in IDC, and 34.68% in IDC-L, and these results followed the progression of BC. This may be the result of chance, or cyclic methylation modification mechanisms might be present, as reported in the prior studies [52, 53], in the *RARβ* promoter region, and these must be clarified in the future.

Finally, by performing an unsupervised clustering analysis of DCIS, IDC, and IDC-L, we found that luminal, Her-2, and triple-negative tumors had different methylation profiles. We synchronously clustered at least three methylation genes with different functions in a single specimen, relative to BC subtypes. The highest methylation frequencies were usually observed in luminal tumors. Her-2 and triple-negative BC samples displayed low methylation frequencies in general, and this result may be compatible with results indicating they have unstable and aberrant genomes, which may result from reduced transposon silencing. The association between methylated modification profiles and different subtypes has been mentioned in many previous investigations [40, 54, 55] because it could indicate that different molecular subtypes of BC could be caused by distinct genetic and epigenetic mechanisms [56].

Abbreviations

GWAS: Genome-wide association studies
CGI: CpG islands
cfDNA: Circulating free DNA
DCIS: Ductal carcinoma in situ
IDC: Invasive ductal carcinoma
IDC-L: Invasive ductal carcinoma plus lymph metastasis
HMEC: Human mammary epithelial cells
ERDN: Early Detection Research Network
TNBC: Triple-negative BC.

Data Availability

(i) The following data (DATA TYPE) used to support the findings of this study are included within the article. The data are as follows: (1) methylation frequencies for the nine genes in sporadic and hereditary BC patients, (2) methylation frequencies of the same genes in the matched serum and methylation-positive BC tissues, (3) different methylation frequencies of HOXD13 and hMLH1 during the BC progression, (4) different methylation frequencies of HOXD13 and WNT5a between sporadic and hereditary BC, (5) DNA-methylated modification levels in different molecular subtypes of BC, and (6) ROC curve analysis of the three-gene methylation panel between BC, age-matched healthy and benign samples. (ii) The following data (DATA TYPE) used to support the findings of this study are included within the supplementary information file(s). The data are as follows: (1) the BSP PCR reaction primers and the system used to analyze the twelve genes., (2) MethyLight primers, probes, and the system used to analyze the twelve genes, (3) the signaling pathways of the candidate genes, (4) primers, probe sequences, and tested methylation sites for all genes, (5) clinicopathologic parameters of patients with BC in the test and validation sets, (6) methylated frequencies of nine genes in BC tissues from the test and validation sets, (7) methylation frequencies for the nine genes in BC patients, (8) the list of coexisting methylated genes in specimens with different histopathological types in sporadic and hereditary BC, (9) BC specimens in which at least three genes were simultaneously methylated in different subtypes (DCIS, IDC, IDC_L, and HpBC), (10) overview of the analyzed procedure, (11) the BSP analysis and the methylated sites chosen for all of the genes, (12) cluster analysis of methylated genes by BC subtypes in different groups ((a) DCIS, (b) IDC, (c) IDC-L, and (d) HpBC), and (13) differential expression of PCDHGB7 between BC tissues and matched normal breast tissues.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ming Shan, Lei Zhang, and Yang Liu contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplemental Table 1: primers, probe sequences, and tested methylation sites for all genes. Supplemental Table 2: clinicopathologic parameters of patients with breast cancer in the test and validation sets. Supplement Table 3: clinical pathological characteristics of benign and normal control cases. Supplemental Table 4: methylated frequencies of nine genes in breast cancer tissues from the test and validation sets. Supplemental Table 5: methylation frequencies for the nine genes in breast cancer patients. Supplemental Table 6: the list of coexisting methylated genes in specimens with different histopathological types in breast cancer. Supplemental Figure 1: overview of the analyzed procedure. Supplemental Figure 2: the BSP analysis and the methylated sites chosen for all of the genes. Supplemental Figure 3: differential expression of PCDHGB7 between BC tissues and matched normal breast tissues. *(Supplementary Materials)*

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Research Article

Loss of SDC1 Expression Is Associated with Poor Prognosis of Colorectal Cancer Patients in Northern China

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Background. Syndecan-1 (SDC1/CD138) is a key cell surface adhesion molecule essential for maintaining cell morphology and the interactions with the surrounding microenvironment. SDC1 tumor immunoexpression may be increased or decreased in epithelial malignant neoplasms compared to that in adjacent non-neoplastic tissue, depending on the type of carcinoma, and it has been correlated with various clinicopathological parameters and patient prognosis. SDC1 expression is decreased in colorectal cancer (CRC) tissue, but the relationship between prognosis and SDC1 expression in CRC patients is controversial. *Methods*. In this study, SDC1 expression was detected in 65 adjacent non-neoplastic colorectal tissues, 477 CRCs, and 79 metastatic lymph nodes using tissue microarray. *Results*. The data show that SDC1 decreased in CRC tissues ($p \leq 0.001$) and metastatic lymph node tissues ($p \leq 0.001$) compared to that in adjacent non-neoplastic colorectal tissues. Loss of SDC1 protein expression is associated with poor overall ($p < 0.0001$) and disease-free survival ($p < 0.0001$), differentiation ($p = 0.017$), stage ($p \leq 0.001$), and lymph node metastasis ($p \leq 0.001$) in CRC patients. *Conclusions*. These data suggest that the loss of SDC1 plays an important role in CRC malignant progression. Loss of SDC1 expression indicates poor prognosis in patients from northern China with CRC.

1. Introduction

Colorectal cancer (CRC) is the most common tumor of the gastrointestinal system and ranks as the fourth leading cause of cancer-related deaths [1]. The highest incidence rates of CRC are observed in Europe, North America, and Oceania; the lowest rates are reported in Asia, Africa, and South America [2].

SDC1 (syndecan-1, CD138), an important cell adhesion molecule, belongs to the family of syndecans, which are transmembrane heparan sulfate proteoglycans (HSPG) [3]. SDC1 is expressed predominantly in epithelial cells, but it is also found in fibroblasts, myoblasts, and differentiating B cells [4–6]. SDC1 can be cleaved and thus releases the extracellular (ectodomain) core protein-shed SDC1 [7]. The shed SDC1 is increased in response to growth factors, chemokines, heparanase, microbial toxins, insulin, and cellular stress [8, 9]. Although the high shed SDC1 levels in serum have been associated with poor prognosis of CRC patients [10], the relationship between prognosis and epithelial SDC1 expression levels in CRC is controversial [4–6].

In this study, SDC1 expression was detected in 65 adjacent non-neoplastic colorectal tissues, 477 CRC tissues, and 79 metastatic lymph node tissues. The aim of this study was to evaluate the relationship between SDC1 expression and the prognosis of CRC patients from China.

2. Materials and Methods

2.1. Colorectal Biopsy Specimens. A cohort of 477 (477/621, 76.8%) subjects with CRC, 65 (65/621, 10.5%) adjacent non-neoplastic colorectal epithelia control subjects, and 79 (79/621, 12.7%) subjects with metastatic lymph nodes were recruited between 2008 and 2014 from the Department of

Gastrointestinal Surgery in the Affiliated Hospital of Jining Medical University (Shandong, PR China). Of the 477 CRC patients, 250 (52.4%) were male and 227 (47.6%) were female (with a mean age of 61 years). All biopsies were immediately fixed in 4% buffered paraformaldehyde and were routinely processed. Tumors were classified according to the standard TNM staging guidelines of UICC (TNM Classification of Malignant Tumours Eighth Edition). All patients had long-term follow-up results. A cohort of 8 fresh CRC biopsies and paired, adjacent non-neoplastic colorectal tissue samples were collected from patients from the Affiliated Hospital of Jining Medical University. The study protocol was reviewed and approved by the local ethics committee. All patients gave written consent for the tissue samples.

2.2. TMA Construction. Representative areas of the CRC, adjacent non-neoplastic colorectal epithelia, and metastatic lymph node tissues were marked on each hematoxylin-eosin (H&E) slide. The TMAs were assembled with a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA) as described by Kallioniemi et al. [11].

2.3. Immunohistochemical Staining. Immunohistochemical staining of the SDC1 protein was performed on the TMA slides using the streptavidin-peroxidase (S-P) method as previously described [12]. Briefly, each TMA section was deparaffinized and rehydrated. Antigen retrieval was performed at 95°C in 1x EDTA (ethylenediaminetetraacetic acid) buffer (pH 9.0) for 15 min. Inactivation of endogenous peroxidase was performed by using 0.3% H_2O_2-methanol for 30 min. Nonspecific binding was prevented by incubation with normal serum for 20 min at room temperature (RT), followed by incubation with the primary monoclonal antibody against human SDC1 (dilution 1 : 100, Clone No. MI15, Fuzhou Maixin Biotech. Co. Ltd., China) at 4°C overnight. Antibody binding was detected using EnVision reagents (Dako REAL EnVision Detection System; peroxidase/DAB1, DakoCytomation, Denmark). The immune reaction was visualized by incubation with 3,30-diaminobenzidine chromogen substrate (DAB1 Chromogen, DAKOVR, Carpinteria, CA, USA) for 10 min at RT. Finally, slides were counterstained with hematoxylin-eosin, dehydrated, and coverslipped with a mounting automat (Sakura GLC 550, Tissue-TekVR, Alphen aan den Rijn, The Netherlands). SDC1 expression was scored by two independent pathologists without prior knowledge of patients' clinicopathological characteristics. Three nonmetastatic lymph nodes were used as negative (T and B cells) and positive (plasmocytes) controls for SDC1 staining. The color photomicrographs were taken with an upright metallurgical microscope. In tumors (adjacent non-neoplastic glandular epithelium and metastatic lymph nodes), immunohistochemical reactions were classified for intensity as previously described [13, 14]. Briefly, low expression (L, - or ±), no staining (-), weak staining (±), or strong staining was observed in less than 25% of tumor cells. Moderate expression (M, +), moderate staining, or strong staining was observed in only 25-75% of tumor cells. High expression (H, ++) and strong staining were observed in more than 75% of tumor cells.

2.4. Protein Extraction and Western Blot. Fresh CRC samples and paired, adjacent non-neoplastic colorectal tissues were homogenized in RIPA lysis buffer (Solarbio, Beijing, China) containing phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Corporation, St Louis, MO, USA). Equal amounts of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were immunoblotted with the following antibodies: monoclonal anti-SDC1 (dilution 1 : 1000, Clone No. 4H5H5) and anti-GAPDH antibodies (dilution 1 : 3000, Proteintech Group Inc., Chicago, IL, USA). The immunoreaction was visualized with enhanced chemiluminescence solution (Millipore, Billerica, MA, USA).

2.5. RNA Isolation and Quantitative Real-Time PCR (qRT-PCR). Total RNA was isolated from fresh CRC samples and paired, adjacent non-neoplastic colorectal tissues using TRIzol Reagent (Invitrogen, San Diego, CA, USA) and then treated with DNase (Roche Diagnostics, Rotkreuz, Switzerland) to eliminate contaminating DNA. Next, 1 μg of the total RNA sample was reverse-transcribed into cDNA using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA). qRT-PCR was performed using a Bio-Rad iQ SYBR Green Supermix kit and the Bio-Rad iCycler iQ system (Bio-Rad, Hercules, CA, USA). Human SDC1 primers were used with the forward sequence (5′-3′) TGGGGATGACTCTGACAACT and the reverse sequence (5′-3′) CACTTCTGGCAGGACT ACAG. Human GAPDH primers were used with the forward sequence (5′-3′) AACGGATTTGGTCGTATTGG and the reverse sequence (5′-3′) TTGATTTTGGAGGGATCTCG. The expression levels of amplified genes were normalized to GAPDH and were presented as relative expression levels.

2.6. Statistical Analysis. Pearson's χ^2 test was used to analyze the association between SDC1 expression and clinicopathological characteristics by using the SPSS 13.0 software package (SPSS, Chicago, IL). The Kaplan-Meier method was used to determine the probability of survival, and GraphPad Prism software (version 6, La Jolla, CA, USA) was used to analyze the data with the log-rank test. Differences in quantitative variables between groups were analyzed by Student's t-test and Mann-Whitney test (nonparametric text, data do not assume Gaussian distributions). In the analyses, a p value of <0.05 was considered significant.

3. Results

3.1. SDC1 Expression Decreased in CRCs and Metastatic Lymph Nodes. We measured SDC1 protein levels by Western blot and mRNA levels by qRT-PCR in 8 fresh CRC samples and paired, adjacent non-neoplastic colorectal tissues. As shown in Figure 1, three expression forms of SDC1 protein were detected, SDC1 protein dimer expression was high (the main expression form), tetramer expression was weak, and monomer expression was absent in all the samples (Figure 1(a)). SDC1 protein was highly expressed in adjacent

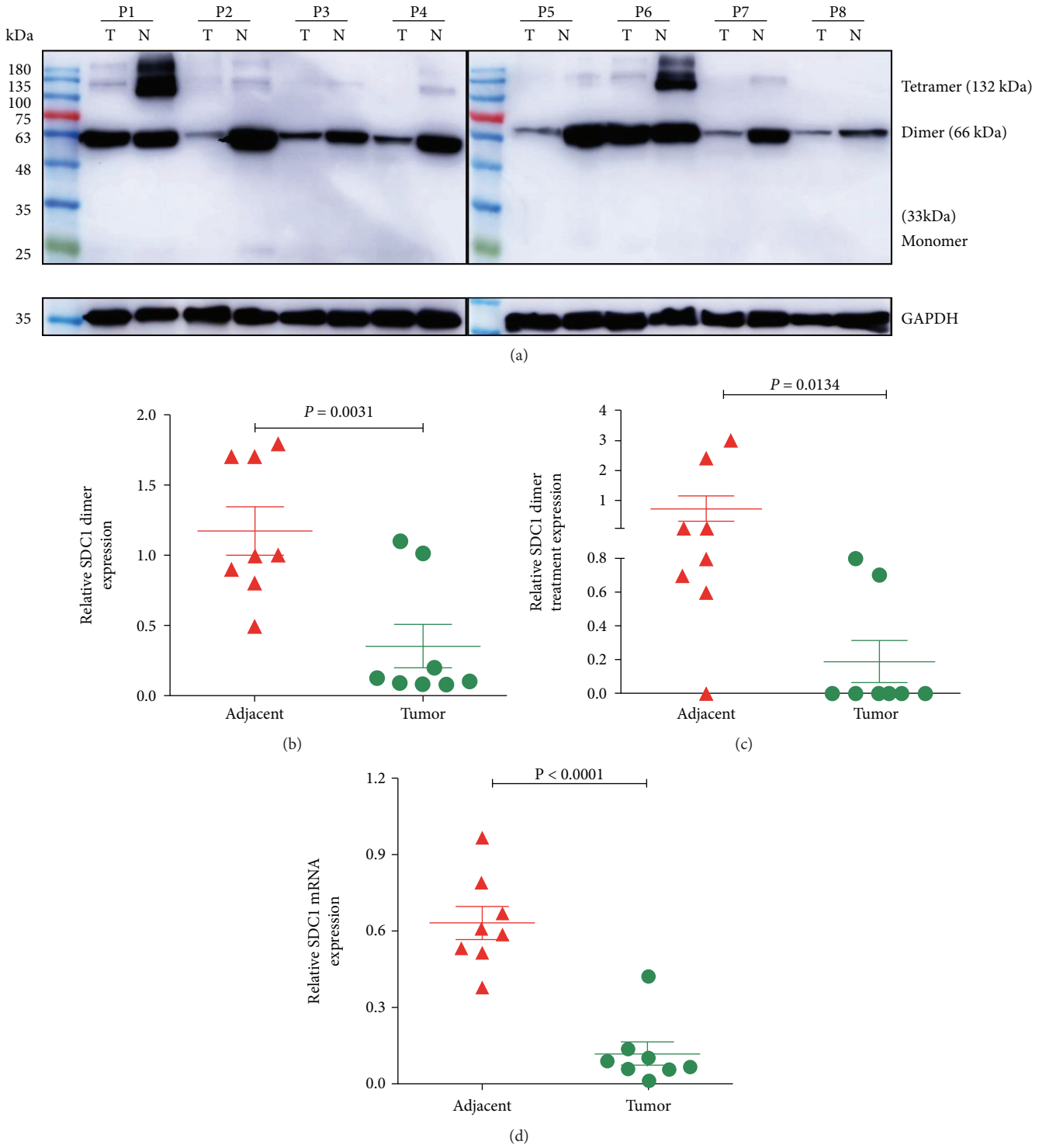

Figure 1: SDC1 expression is decreased in CRCs. (a) Protein expression levels were evaluated in colorectal biopsies taken from 8 tumors (CRCs, T) and adjacent non-neoplastic controls (N) by Western blot. GAPDH was used as a loading control. Quantitative analysis of SDC1 protein: (b) dimer level ($p = 0.0031$, Student's t-test) and (c) tetramer level ($p < 0.0001$, Mann-Whitney test) in CRC and paired, adjacent non-neoplastic controls. (d) mRNA expression levels were evaluated in colorectal biopsies and adjacent non-neoplastic controls by qRT-PCR. GAPDH was used as a control. $p < 0.0001$, Student's t-test.

non-neoplastic colorectal tissue homogenates but was weak or undetectable in CRC tissues (Figure 1(b), dimer, $p = 0.0031$; Figure 1(c), tetramer, $p = 0.0134$); SDC1 mRNA expression was decreased in CRC tissues compared to that in paired, adjacent non-neoplastic colorectal tissues (Figure 1(d), $p < 0.001$).

Furthermore, we detected SDC1 expression in 621 cases: 477 samples of CRCs (Figure 2(a), high expression;

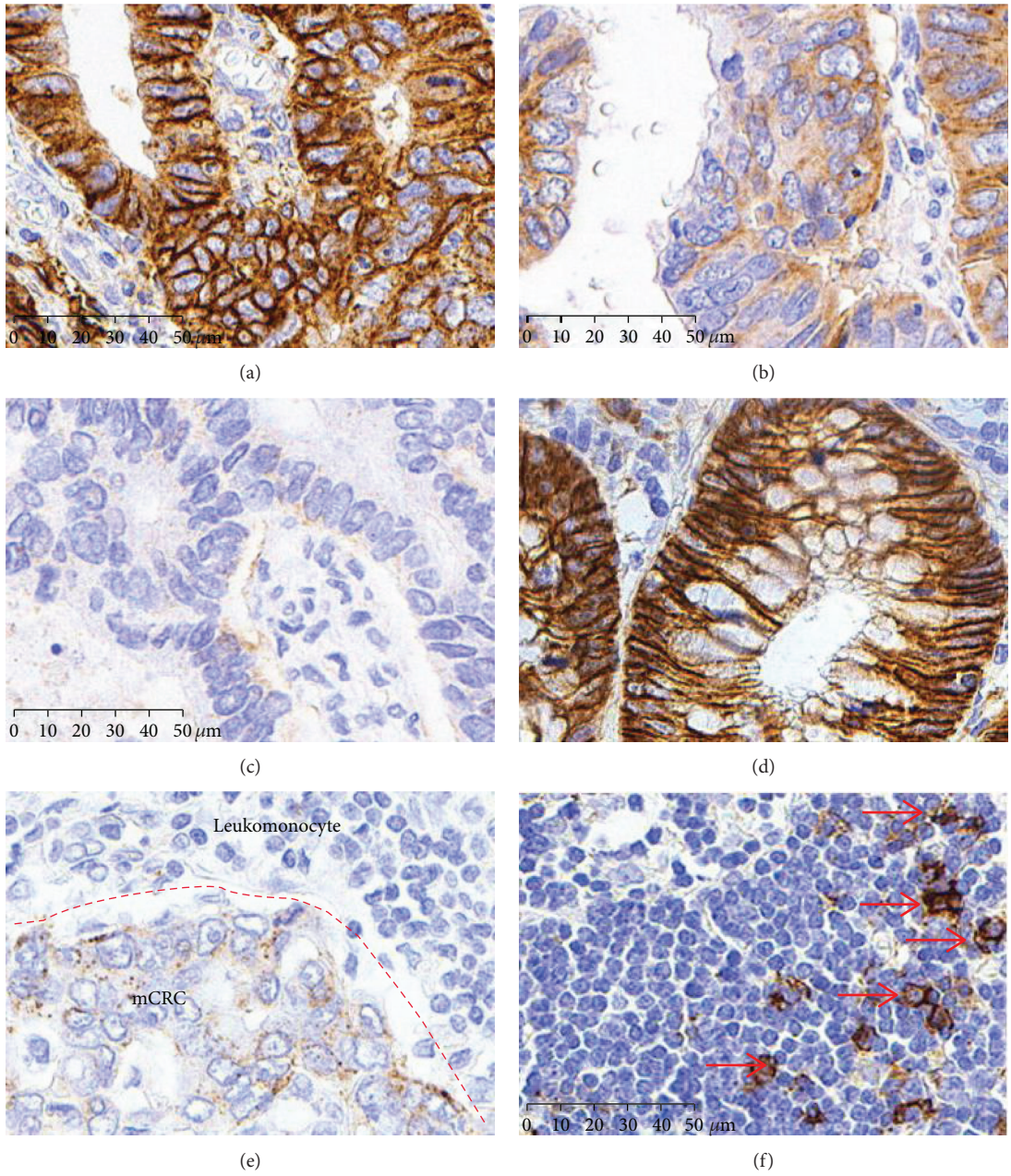

Figure 2: SDC1 decreased both in CRCs and metastatic lymph nodes (mCRC). Immunohistochemical staining for SDC1 in CRCs. (a) High expression, (b) moderate expression, (c) low expression, (d) adjacent non-neoplastic colonic epithelium (high expression), and (e) metastatic lymph nodes (low expression). (f) SDC1 staining in nonmetastatic lymph node as negative control (nonstaining cells, T and B cells) and positive control (red arrow, plasmocytes). Scale bars: 50 μm.

2(b), moderate expression; and 2(c), low expression), 65 samples of adjacent non-neoplastic colorectal epithelium (Figure 2(d)), and 79 samples of metastatic lymph nodes (Figure 2(e)) from CRCs by immunohistochemistry (IHC). IHC revealed that high SDC1 expression in adjacent non-neoplastic colorectal epithelial cells was detected mainly in the membrane and cytoplasm (Figure 2(d)). In contrast, SDC1 expression was reduced or undetectable in CRC tissues and metastatic lymph nodes from CRCs. Statistically, among the 65 adjacent non-neoplastic colorectal epithelium samples, 57 (88%) samples showed high expression of SDC1, and 8 (12%) samples showed moderate expression of SDC1. Only 109 of 477 (22.8%) tissues and 228 of 477 (44.8%) CRC samples exhibited high or moderate SDC1 expression, respectively. The immunointensity of SDC1 in metastatic lymph nodes from CRCs further decreased to lower levels since only 11 of 79 (14%) samples showed high SDC1 expression (Table 1). Thus, these data indicate that the loss of SDC1 expression is involved in the development and progression of CRC in northern China.

3.2. Association between SDC1 Expression in CRC Tissues and Patients' Clinicopathological Characteristics. According to the SDC1 staining intensity and the extent of positive tumor

Table 1: Loss of SDC1 expression in CRCs and metastatic lymph nodes, 621 cases.

	$SDC1^L$ $N = 180$ Case (%)	$SDC1^M$ $N = 264$ Case (%)	$SDC1^H$ $N = 177$ Case (%)	Total	χ^2	p
Adjacent non-neoplastic tissues[a]	0 (0)	8 (12)	57 (88)	65	114.154	$\leq 0.001^{ab}$
CRCs[b]	140 (29.4)	228 (47.8)	109 (22.8)	477	151.685	$\leq 0.001^{bc}$
Metastatic lymph nodes[c]	40 (51)	28 (35)	11 (14)	79	81.639	$\leq 0.001^{ac}$

CRC: colorectal cancer; L: low expression; M: moderate expression; H: high expression; statistical method: chi-square test.

Table 2: Relationship between SDC1 immunoreactivity and clinicopathological characteristics, 477 CRC cases.

Clinical information	Total	$SDC1^L$ $N = 140$ Case (%)	$SDC1^M$ $N = 228$ Case (%)	$SDC1^H$ $N = 109$ Case (%)	χ^2	p
Age, yr						
<60.5	211	67 (32)	93 (44)	51 (24)	2.13	0.345
>60.5	266	73 (27)	135 (51)	58 (22)		
Gender						
Female	227	69 (30)	110 (49)	48 (21)	0.752	0.686
Male	250	71 (28)	118 (47)	61 (24)		
Tumor size, cm						
≤4	306	92 (30)	138 (45)	76 (25)	2.924	0.232
>4	171	48 (28)	90 (53)	33 (19)		
Histological grade (differentiation) (miss samples, N = 16)						
Well	226	75 (33)	111 (49)	40 (18)	8.12	0.017
Moderately or poorly	235	59 (25)	110 (47)	66 (28)		
TNM stage (T)						
1-2	69	14 (20)	22 (32)	33 (48)	28.55	≤0.001
3-4	408	126 (31)	206 (50)	76 (19)		
Lymph node metastasis						
N_0	290	66 (23)	140 (48)	84 (29)	23.088	≤0.001
N_{1-3}	187	74 (40)	88 (47)	25 (13)		
Location (miss samples, N = 286)						
Left	49	17 (35)	23 (47)	9 (18)	1.123	0.570
Right	142	38 (27)	74 (52)	30 (21)		

L: low expression; M: moderate expression; H: high expression; statistical method: chi-square test.

cells, our data showed that SDC1 was expressed at low levels in 29.4% (140/477) of CRCs, moderately expressed in 44.8% (228/477) of CRCs, and highly expressed in 22.8% (109/477) of CRCs (Table 1). We next assessed the relationship between SDC1 expression and patients' clinicopathological characteristics. SDC1 expression was not correlated with age ($p = 0.345$), sex ($p = 0.686$), or tumor diameter ($p = 0.232$). However, loss of SDC1 protein was significantly associated with poor differentiation ($p = 0.017$), advanced TNM stage ($p \leq 0.001$), and LN metastasis ($p \leq 0.001$) (Table 2).

3.3. Loss of SDC1 Expression Is Significantly Associated with Poor Prognosis of ESCC Patients. Kaplan-Meier analysis revealed that patients who exhibited reduced SDC1 (moderate or low) expression were associated with poorer overall survival and disease-free survival compared to patients who exhibited high SDC1 expression ($p = 0.0045$, $p < 0.0001$ and $p = 0.0038$, $p < 0.0001$, respectively, Figures 3(a) and 3(b)). These data indicate that SDC1 plays a role as a reliable tumor suppressor in CRC.

4. Discussion

Our findings derive from a large, clinically annotated tissue microarray of CRC specimens and add to the body of evidence that the loss of epithelial SDC1 is a general feature of carcinoma progression. The loss of SDC1 expression in local lymph node metastasis is evidence of the prometastasis function of SDC1. In fact, our results showed that the loss of expression of epithelial SDC1 truly correlates with poor dedifferentiation, stage, and local lymph node metastasis in CRC. In agreement with other analyses of CRC, the loss of epithelial SDC1 was correlated with tumor TNM

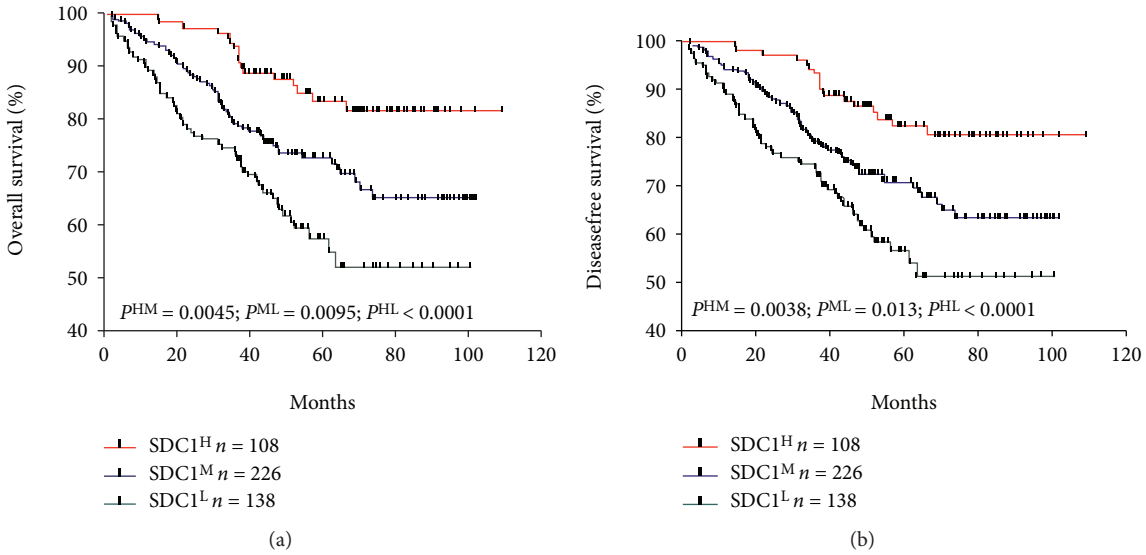

Figure 3: Relationship between CRC SDC1 status and patient survival. The Kaplan-Meier survival curves demonstrate that low, moderate, and high SDC1 expression in the tumors did correlate with (a) overall survival and (b) disease-free survival.

stage [4–6, 13], and the incidence of metastasis was correlated with local lymph nodes [4–6].

SDC3 and SDC4 have been reported as oncogenes [15, 16]. Recently, syndecan-2 (SDC2) methylation was highlighted as a potential marker for early CRC detection. A DNA microarray analysis of neoplastic samples showed a high SDC2 methylation rate of approximately 95%, regardless of the early colorectal cancer stage [17]. Blood SDC2 methylation data from 131 CRC patients and 125 healthy subjects showed a high sensitivity of 92.3% for detecting stage I CRCs [18]. Bowel lavage fluid (BLF) SDC2 methylation data showed that SDC2 methylation was positive in 100% of villous adenoma, high-grade dysplasia, and hyperplastic polyp samples; in 88.9% of tubular adenoma samples; and in 0% of normal mucosal samples [19]. These results suggest that the reduction in SDC1 expression in CRCs may also be caused by *SDC1* DNA methylation, and further research is needed.

The syndecan transmembrane domain and transmembrane domain-induced dimerization seem to critically regulate various functions of syndecan family members [20]. Research has shown that SDC1 is coexpressed with EMT markers (E-cadherin and β-catenin) in CRCs and that this coexpression is regulated during epithelial-mesenchymal transition (EMT) [21]. The loss of SDC1 expression in carcinoma cells reduces cell adhesion to the extracellular matrix and enhances cell motility and invasion [22]. Our results showed that SDC1 expression was mainly in the form of a dimer in normal colorectal epithelial cells and was downregulated in CRCs. This suggests that SDC1 is inactivated in CRCs, thus reducing cell adhesion to the extracellular matrix and enhancing cell motility and invasion.

We also found that the loss of expression of epithelial SDC1 significantly correlates with poor patient survival. Previous studies reached conflicting conclusions on whether reduced SDC1 is correlated with decreased patient survival [4–6]. A study from Japan revealed that the low expression of epithelial SDC1 was significantly associated with poor clinical outcome in CRC [11], but two studies from Finland and the USA have shown that the low expression of epithelial SDC1 did not significantly correlate with the survival of CRC [23, 24]. It is important to note that the studies that have examined the use of SDC1 as a prognostic marker were performed in different countries and on different continents [4–6]. Therefore, other factors such as treatment plans, genetic variations, and ethnicity may have influenced the results and affected the prognostic value of SDC1 in CRC progression and metastasis. Our data are in agreement with data from the study of Fujiya et al. [14]. The data suggest that the relationship between SDC1 expression and the prognosis of CRC patients may have ethnic and regional differences: the loss of SDC1 expression was correlated with a poor prognosis for East Asian CRC patients but not for Europeans.

In summary, the loss of SDC1 expression in CRC is closely associated with poor differentiation, stage, and local lymph node metastasis. SDC1 is a valuable biomarker for predicting the prognosis of CRC patients in northern China.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Association between *AXIN1* Gene Polymorphisms and Bladder Cancer in Chinese Han Population

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Background. Previous evidence has indicated that the reduction of axis inhibition protein 1 (*AXIN1*) expression is related with the poor differentiation of non-small-cell lung cancer (NSCLC). However, the potential association between AXIN1 and bladder cancer (BC) is unknown. We aimed to initially explore the relevance of *AXIN1* gene polymorphisms (rs12921862 C/A, rs1805105 T/C, and rs370681 C/T) and BC. *Methods*. Three hundred and sixteen BC patients and 419 healthy controls had been enrolled. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping three tag single-nucleotide polymorphisms (SNPs) of *AXIN1*. The SNPstats online analysis software and SPSS software were used for statistical analysis. *Results*. Our data revealed that three tag SNPs were associated with an increased risk of BC (rs12921862: $P < 0.001$, OR (95%CI) = 4.61 (3.13-6.81); rs1805105: $P = 0.046$, OR (95%CI) = 1.35 (1.00-1.82); and rs370681: $P = 0.004$, OR (95%CI) = 1.56 (1.15-2.10)). For rs12921862, A allele was an independently protective factor which correlated with a better prognosis in non-muscle-invasive bladder cancer (NMIBC) patients ($P = 0.03$, OR (95%CI) = 0.10 (0.01-0.84)). Stratification analysis demonstrated that rs370681 polymorphism was related with high-grade bladder cancer ($P = 0.04$, OR (95%CI) = 1.85 (1.04-3.23)). *Conclusion*. The *AXIN1* gene polymorphisms might implicate in BC risk, and rs12921862 could be a potential forecasting factor for prognosis of BC patients.

1. Introduction

Bladder cancer (BC) was ranked as the ninth most common cancer by the International Agency for Research on Cancer in 2012. It is related to thrice as much higher morbidity and mortality in the developed countries compared to the developing regions, and more than 75% of the cases occur in men. In 2012, 165000 deaths and 429000 new cases were recorded worldwide, of which 26820 deaths and 55486 new cases were in China, resulting in an incidence rate of 3/100000 and accounting for a large fraction of BC in East Asia (37491 deaths and 85451 cases) [1]. Around 80% of BC patients have the non-muscle-invasive bladder cancer (NMIBC), whereas muscle-invasive bladder cancers (MIBC) account for only 20% of the cases, although they are responsible for a large number of deaths [2–4].

Smoking is the most significant risk factor of BC, accounting for an estimated 50% of all cases, followed by occupational exposure to chemical carcinogens, e.g., industrial paints, dyes, metals, and petroleum products [3–5]. Increasing evidences in recent years have suggested a genetic predisposition towards cancer susceptibility. For instance,

Gu et al. found an association between the N-acetyl transferase 2 (NAT2) slow acetylator phenotype and a significantly higher risk of BC in smokers [6]. Moreover, the risk of BC is twofold higher in first-degree relatives of BC patients [4], indicating that several genetic factors could play a role in the initiation and progression of BC.

The Wnt/β-catenin signaling pathway is one of the fundamental pathways regulating cell proliferation, polarity, and lineage differentiation during embryonic development and tissue homeostasis, and mutations in its components are often involved in birth defects, cancer, and other diseases [7, 8]. Studies have directly linked the Wnt signaling pathway to the development of BC [9–11]. Mao et al. demonstrated that activating the Wnt pathway could accelerate the epithelial-mesenchymal transition (EMT), invasion, and migration of BC cells *in vitro* [12]. In addition, the inhibition of Wnt signaling suppressed BC xenograft growth in nude mice [13]. Canonical Wnt signaling is regulated by the degradation of the AXIN1-mediated β-catenin destruction complex. Axis inhibition protein 1 (AXIN1) is a multidomain scaffold protein that regulates the levels and localization of β-catenin during Wnt pathway activation and is involved in the genesis and progression of diseases like atrial septal defect, cryptorchidism, caudal duplication anomalies, breast cancer, non-small-cell lung cancer (NSCLC), hepatitis B virus-related hepatocellular carcinoma (HCC), colorectal cancer, and gastrointestinal cancer [14–22].

AXIN1, the gene encoding AXIN1, is located within a 65 kb region on chromosome 16p. AXIN1 acts as a tumor suppressor, and mutations in this protein have been shown to play a significant role in carcinogenesis [23]. However, no link has been established so far between AXIN1 and BC. The aim of our study was to determine whether the polymorphic variants of *AXIN1* contribute to BC susceptibility. We selected three tag single-nucleotide polymorphisms (SNPs)—rs12921862, rs1805105, and rs370681—and determined their prevalence in 316 unrelated BC patients and 419 healthy controls in a Chinese Han population.

2. Material and Methods

2.1. Participants' Clinical Characteristics and Follow-Up. We enrolled 316 patients with bladder cancer (mean age ± SD: 63.76 ± 12.14) and 419 healthy controls (mean age ± SD: 59.88 ± 11.32) from the West China Hospital of Sichuan University from 2007 to 2012. A case control study based on the hospital was approved by the hospital ethics committee, and informed consents were provided by all the participants. Follow-up data of participants were abstracted by telephone calls every 6 months for 5 years. Histopathological analysis was used to confirm the tumor tissues from resected specimens of patients, and the clinical characteristics are summarized in Table 1. This study excluded the control participants who had a personal or family history of BC or other severe diseases and the patients who had previous cancer or metastasized cancer from other origins as well as underwent radiotherapy or chemotherapy. All participants were genetically unrelated individuals of the Han population living in Sichuan province of China.

Table 1: Characteristics of the studied population.

Characteristics	Patients	Controls
Age at first diagnosis (mean ± SD)	63.76 ± 12.14	59.88 ± 11.32
Sex		
Male	250 (79.1%)	225 (53.7%)
Female	66 (20.9)	194 (46.3%)
Smoking status		
Smoker	163 (51.6%)	184 (43.9%)
Nonsmoker	153 (48.4%)	235 (56.1%)
Tumor stage		
MIBC	149 (47.2%)	
NMIBC	167 (52.8%)	
Tumor grade		
High grade	182 (57.6%)	
Low grade	134 (42.4%)	
Clinical stage		
I (Ta ~ T1N0M0)	156 (53.1%)	
II (T2N0M0)	84 (28.6%)	
III (T3N0M0, T4aN0M0)	34 (11.6%)	
IV (T4bN0M0, TnNnM0, TnNnMn, $n \geq 1$)	20 (6.7%)	

2.2. AXIN1 Genotyping. The three tag SNPs were picked out according to data in the CHB population sample of the HapMap Project (Data Release 24/Phase II, NCBI build 36 assembly, dpSNPb126) using the algorithm-Tagger-pairwise Tagging from the international HapMap Project [24]. And the PCR primers were designed with software Primer 3 web version 4.1.0. (http://primer3.ut.ee/) [25] as shown in Table 2 (in this part, we followed the methods of Li et al. [26]).

DNA isolation kit from BioTeke (Peking, China) was used to extract each individual's genomic DNA from a 200 μL EDTA-anticoagulated peripheral blood sample. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The DNA fragments containing the polymorphisms were amplified in a total volume of 10 μL, including 5 μL of 2x power Taq PCR Master Mix (BioTeke, Peking, China), 2.7 pico mole of each primer, and 100 ng genomic DNA for rs12921862 and rs1805105, and a total volume of 25 μL, including 2.5 μL of Taq Buffer, 0.3 μL Taq enzyme (BioTeke, Peking, China), 3 μL DNTP, 2.7 pico mole of each primer, and 100 ng genomic DNA for rs370681. The PCR conditions were 94°C for 4 min, followed by 34 cycles of 30 s at 94°C, 30 s at 62°C, 65°C, or 66°C, respectively, and 30 s at 72°C, with a final elongation at 72°C for 10 min for three tag SNPs. Following the termination of PCR, the PCR products were digested by restriction enzyme (presented in Table 2). Finally, the digested fragments were separated by a 6% polyacrylamide gel and stained with 1.5 g/L argent nitrate. Furthermore, the genotypes were confirmed by DNA sequencing analysis. About 10% of the samples were randomly selected to perform the repeated assays, and the results were 100% in agreement.

Table 2: Primer sequences and reaction conditions for genotyping three tag SNPs in *AXIN1* gene.

SNPs	Primer sequence	Major/minor gene	Annealing temperature (°C)	Restriction enzyme	Product size (bp)
rs12921862	F: 5′-CTCACGCCAGTGCCTCTACT-3′ R: 5′-ATGCCATCCATGTGGAAACT-3′	C/A	62	*ScrF I*	A: 216 C: 110 + 106
rs1805105	F: 5′-CTGGATACCTGCCGACCTTA-3′ R: 5′-ACCTTTCCCTGGCTTGTTCT-3′	T/C	65	*Fok I*	C: 245 T: 186 + 59
rs370681	F: 5′-GAGGCCTAAGCTCCAGGCACT-3′ R: 5′-AAGGAAAGTGGGTTCTCCACCCA-3′	C/T	66	*Btsα I*	T: 166 C: 150 + 16

2.3. Statistical Analyses. SPSS for Windows software package version 20.0 (SPSS Inc., Chicago, IL, USA) was used to analysis the data. Genotypic association were provided by the SNPstats online analysis software including the codominant, dominant, recessive, and overdominant genetic models [27], and the Hardy-Weinberg equilibrium was evaluated by chi-squared test. The level of significance was set at $P < 0.05$. The effects of different genotypes and alleles were evaluated by odds ratio (OR) and respective 95% confidence intervals (95% CI). Kaplan-Meier plots and the log-rank test were used to estimate the relationships of *AXIN1* genotypes with patients' outcomes (recurrence and death). Considering age at first diagnosis, sex, smoking status, tumor grade, and clinical stage, Cox regression analysis model was used for multivariate survival analysis.

3. Results

3.1. AXIN1 SNP Frequencies and BC Susceptibility. The distributions of the three tag SNPs' genotypes and alleles in both case and control groups were in agreement with the Hardy-Weinberg equilibrium ($P > 0.05$). The differences in the genotypes and allele frequencies of *AXIN1* SNPs between patients and controls are shown in Table 3. The frequency of the heterozygous genotype (CA) of rs12921862 was significantly higher among the patients compared to controls in the overdominant model (33.5% vs. 8.8%, $P < 0.001$, OR = 5.21, 95% CI = 3.46 − 7.86). In the dominant model, a significantly increased BC risk was related to the CA/AA genotypes (35.1% vs. 10.5%, $P < 0.001$, OR = 4.61, 95% CI = 3.13 − 6.81). Compared to the CC/AA genotypes, the CA genotype was associated with a higher risk of BC in the codominant model ($P < 0.001$, OR = 5.24, 95% CI = 3.47 − 7.91). Similarly, a significant relationship was observed between the A allele-carrying patients and controls (18.4% vs. 6.1%, $P < 0.001$, OR = 3.45, 95% CI = 2.44 − 5.00). For rs1805105, compared with TT carriers, TC/CC carriers were significantly higher in the dominant model (46.5% vs. 39.1%, $P = 0.046$, OR = 1.35, 95% CI = 1.00 − 1.82). In the overdominant model, the TC genotype was significantly associated with increased BC risk compared to the TT/CC genotypes ($P = 0.04$, OR = 1.38, 95% CI = 1.01 − 1.87). For rs370681, significant differences were found in the codominant (CC vs. CT vs. TT, $P = 0.002$, $OR_{CT} = 1.43$, 95% $CI_{CT} = 1.05 - 1.96$, $OR_{TT} = 2.51$, 95% $CI_{TT} = 1.43 - 4.40$), dominant (CC vs. CT/TT, $P = 0.004$, OR = 1.56, 95% CI = 1.15 − 2.10), and recessive models (CC/CT vs. TT, $P = 0.01$, OR = 2.07, 95% CI = 1.21 − 3.55) compared to healthy controls. Furthermore, the frequency of T allele was significantly higher in the BC patients compared to the healthy controls ($P = 0.001$, OR = 1.46, 95% CI = 1.16 − 1.82).

3.2. Clinical Characteristics of the SNP Genotypes. To gain further insights into the association between the three tag SNPs and BC, the patients with different SNP genotypes were stratified (Table 4) according to age (≤64 and >64 years old), sex (male and female), smoking status (smoker and nonsmoker), tumor stage (MIBC and NMIBC), tumor grade (high grade and low grade), recurrence status (recurrent and nonrecurrent), and metastasis status (metastatic and nonmetastatic). No significant difference was observed for any subgroups of the three tag SNPs except age and tumor grades. The CA/AA genotypes of rs12921862 had a significantly higher percentage of patients older than 64 years compared with those under 64 years (40.1% vs. 29.2%, $P = 0.04$, OR = 1.64, 95% CI = 1.01 − 2.70), after adjusting for sex, smoking status, tumor stage, tumor grade, recurrence status, and metastasis status. For rs370681, after adjusting for these common risk factors, the T allele-carrying subgroup had a higher frequency of patients with high-grade tumors compared to those with low-grade tumors (68% vs. 54%, $P = 0.04$, OR = 1.85, 95% CI = 1.04 − 3.23). In contrast, no significant relation was seen between the rs1805105 genotypes and the patients' characteristics after adjusting for common risk factors ($P > 0.05$).

3.3. Prognostic Value of the SNP Genotypes. The statistical association of the SNPs and overall survival of BC patients is summarized in Table 5. At the end of the follow-up in our study, 51 patients (51/316; 16.1%) including 38 MIBC patients and 13 NMIBC patients had died and 93 patients (93/316; 29.4%) including 46 MIBC patients and 47 NMIBC patients had relapsed. Kaplan-Meier survival analysis indicated worse prognosis of BC patients with the rs12921862 AA homozygote (log-rank: $P = 0.003$, Figure 1). However, no significant relationship was observed between the overall survival and the other two SNPs (rs1805105 and rs370681) or between the three SNPs and recurrence-free survival.

Following the stratification of patients by tumor stage (MIBC and NMIBC), we conducted Cox univariate and

Table 3: Distribution of SNPs in *AXIN1* between patients and controls as well as their association with bladder cancer risk.

Model	Genotype	Patients *N* (%)	Controls *N* (%)	Logistic regression OR (95% CI)	*P* value
rs12921862					
Codominant	CC	205 (64.9%)	375 (89.5%)	1.00	
	CA	106 (33.5%)	37 (8.8%)	**5.24 (3.47-7.91)**	**<0.001**
	AA	5 (1.6%)	7 (1.7%)	1.31 (0.41-4.17)	
Dominant	CC	205 (64.9%)	375 (89.5%)	1.00	
	CA/AA	111 (35.1%)	44 (10.5%)	**4.61 (3.13-6.81)**	**<0.001**
Recessive	CC/CA	311 (98.4%)	412 (98.3%)	1.00	
	AA	5 (1.6%)	7 (1.7%)	0.95 (0.30-3.01)	0.93
Overdominant	CC/AA	210 (66.5%)	382 (91.2%)	1.00	
	CA	106 (33.5%)	37 (8.8%)	**5.21 (3.46-7.86)**	**<0.001**
Allele	C	516 (81.6%)	787 (93.9%)	1.00	
	A	116 (18.4%)	51 (6.1%)	**3.45 (2.44-5.00)**	**<0.001**
rs1805105					
Codominant	TT	168 (53.5%)	255 (60.9%)	1.00	
	TC	125 (39.8%)	136 (32.5%)	**1.40 (1.02-1.90)**	0.11
	CC	21 (6.7%)	28 (6.7%)	1.14 (0.63-2.07)	
Dominant	TT	168 (53.5%)	255 (60.9%)	1.00	
	TC/CC	146 (46.5%)	164 (39.1%)	**1.35 (1.00-1.82)**	**0.046**
Recessive	TT/TC	293 (93.3%)	391 (93.3%)	1.00	
	CC	21 (6.7%)	28 (6.7%)	1.00 (0.56-1.80)	1
Overdominant	TT/CC	189 (60.2%)	283 (67.5%)	1.00	
	TC	125 (39.8%)	136 (32.5%)	**1.38 (1.01-1.87)**	**0.04**
Allele	T	461 (73.4%)	646 (77.1%)	1.00	
	C	167 (26.6%)	192 (22.9%)	1.22 (0.96-1.55)	0.11
rs370681					
Codominant	CC	114 (37.9%)	204 (48.7%)	1.00	
	CT	152 (50.5%)	190 (45.4%)	**1.43 (1.05-1.96)**	**0.002**
	TT	35 (11.6%)	25 (6%)	**2.51 (1.43-4.40)**	
Dominant	CC	114 (37.9%)	204 (48.7%)	1.00	
	CT/TT	187 (62.1%)	215 (51.3%)	**1.56 (1.15-2.10)**	**0.004**
Recessive	CC/CT	266 (88.4%)	394 (94%)	1.00	
	TT	35 (11.6%)	25 (6%)	**2.07 (1.21-3.55)**	**0.01**
Overdominant	CC/TT	149 (49.5%)	229 (54.6%)	1.00	
	CT	152 (50.5%)	190 (45.4%)	1.23 (0.91-1.65)	0.17
Allele	C	380 (63.1%)	598 (71.4%)	1.00	
	T	222 (36.9%)	240 (28.6%)	**1.46 (1.16-1.82)**	**0.001**

Boldfaced values indicate a significant difference at the 5% level. OR: odds ratio; CI: confidence interval.

multivariate analyses to determine the predictors of prognosis and survival in the different SNP genotypes (Table 5). Univariate analysis showed no significant association between tumor stage and overall survival in any genotype of the three SNPs in BC patients. However, the multivariate analysis showed a correlation between the CA/AA genotypes of *AXIN1* rs12921862 and significantly higher overall survival rate ($P = 0.03$, OR = 0.1, 95% CI = 0.01 – 0.84), compared to the CC genotype in NMIBC patients. Kaplan-Meier survival analysis reiterated that the CA/AA genotypes were associated with a better prognosis of NMIBC patients (log-rank: $P = 0.03$, Figure 2) compared to the CC genotype.

4. Discussion

The Wnt signaling pathway plays a key role during embryonic development and tissue homeostasis, and its dysregulation is associated with various diseases including cancers [7, 8]. The tumor suppressor function of AXIN1 and AXIN2 proteins in the Wnt signaling pathway has long been hypothesized, based largely on their roles in the β-catenin destruction complex. However, no study had directly linked AXIN1 with BC progression or the proliferation of BC cells. In the present study, we analyzed the potential relationship between different *AXIN1* gene SNPs and BC risk in a

Table 4: Distribution of SNPs in *AXIN1* between patients' characteristics and their association with bladder cancer risk.

Characteristics	rs12921862 Genotype		*P* value[a]	rs1805105 Genotype		*P* value[a]	rs370681 Genotype		*P* value[a]
	CC	CA/AA		TT	TC/CC		CC	CT/TT	
Age									
≤64 years old	102 (70.8%)	42 (29.2%)	***0.04***	85 (59%)	59 (41%)	0.10	56 (40.3%)	83 (59.7%)	0.83
>64 years old	103 (59.9%)	69 (40.1%)		83 (48.8%)	87 (51.2%)		58 (35.8%)	104 (64.2%)	
Sex									
Male	164 (65.9%)	85 (34.1%)	0.77	128 (51.6%)	120 (48.4%)	0.47	88 (37.5%)	147 (62.5%)	0.96
Female	41 (61.2%)	26 (38.8%)		40 (60.6%)	26 (39.4%)		26 (39.4%)	40 (60.6%)	
Smoke									
Smoker	111 (68.1%)	52 (31.9%)	0.4	80 (49.4%)	82 (50.6%)	0.25	58 (37.7%)	96 (62.3%)	0.90
Nonsmoker	94 (61.4%)	59 (38.6%)		88 (57.9%)	64 (42.1%)		56 (38.1%)	91 (61.9%)	
Tumor stage									
MIBC	98 (65.3%)	52 (34.7%)	0.94	74 (49.7%)	75 (50.3%)	0.97	48 (33.1%)	97 (66.9%)	0.44
NMIBC	107 (64.5%)	59 (35.5%)		94 (57%)	71 (43%)		66 (42.3%)	90 (57.7%)	
Tumor grade									
High grade	120 (65.9%)	62 (34.1%)	0.49	87 (48.3%)	93 (51.7%)	0.18	56 (32%)	119 (68%)	***0.04***
Low grade	85 (63.4%)	49 (36.6%)		81 (60.5%)	53 (39.5%)		58 (46%)	68 (54%)	
Recurrence									
Recurrent	59 (63.4%)	34 (36.6%)	0.84	50 (53.8%)	43 (46.2%)	0.97	32 (36%)	57 (64%)	0.40
Nonrecurrent	146 (65.5%)	77 (34.5%)		118 (53.4%)	103 (46.6%)		82 (38.7%)	130 (61.3%)	
Metastasis									
Metastatic	31 (62%)	19 (38%)	0.68	24 (49%)	25 (51%)	0.70	19 (40.4%)	28 (59.6%)	0.07
Nonmetastatic	174 (65.4%)	92 (34.6%)		144 (54.3%)	121 (45.7%)		94 (37.1%)	159 (62.9%)	

Italic values indicate a significant difference at the 5% level. [a]Adjusted by age, sex, smoking status, tumor stage, tumor grade, recurrence status, and metastasis status.

Chinese Han population. Despite extensive linkage disequilibrium in the human genome, a tag SNP could represent most of the haplotypes in a specific region. Our data revealed that the three tag SNPs were associated with an increased risk of BC, which corresponded to the previous observations that activating or inhibiting Wnt signaling accelerates or suppresses, respectively, the invasion and migration of BC cells as well as the carcinogenesis of BC tumor [12, 13]. We calculated the statistical power using Power and Sample Size Calculations for Windows software package version 3.0 [28], and it showed that our data had more than 80% power to detect the relationship between *AXIN1* SNPs and BC risk.

Tumor suppressor protein AXIN1 deregulates β-catenin and mitosis to weaken the ER^+ and ER^- breast cancer [14, 19]. It interacts with β-catenin and regulates its localization, along with that of Wnt-dependent downstream targets like cyclin D1, fra-1, c-myc, and c-jun [18, 29]. One previous study showed that poorly differentiated NSCLC had low levels of AXIN1 relating a high expression of nuclear β-catenin, which was reversed in the well- or moderately differentiated tumors [30]. The oncogenic effects of *AXIN* monoallele mutations that lead to β-catenin stabilization result from the dominant negative activity [31]. Compared to the CC homozygote of the rs12921862 SNP, the CA/AA genotypes were associated with a significantly higher overall survival rate and implicated in a better prognosis of the BC patients, which might indicate a potential dominant negative protective effect of the latter in NMIBC patients. Furthermore, these results are consistent with the better prognosis of NMIBC patients compared to the MIBC.

Tumor stratification is used to grade cancers according to their malignancy: high-grade tumor classification normally represents a worse prognosis compared to that of the low grade. Studies have shown that the *AXIN1* rs1805105 polymorphism is correlated with the early tumor stages (I and II, modified by the Union for International Cancer Control) and the small tumor size (under 5 cm) of HCC [32] but increases the risk of developing advance-stage renal carcinogenesis (RCC III and IV) [33]. However, we found no significant association between the rs1805105 genotypes and tumor grade in our study, although the C allele carriers had a higher (albeit nonsignificant: 51.7% vs. 39.5%) frequency of patients with high-grade tumor compared to those with low-grade tumors. This indicated a nonsignificant tendency that the C allele may be correlated with the potential risk of developing higher-grade tumors among BC patients. Interestingly, the frequency of T allele carriers of the rs370681 SNP with high-grade tumors was significantly elevated than that with low-grade tumors, which indicated an association of this SNP with severe BC. These results are consistent with previous studies on HCC and RCC.

Bladder cancer is an older-susceptible cancer although the average age of the afflicted has decreased over these years. In this study also, we found that the CA/AA carriers of

Table 5: Association between SNPs in *AXIN1* and overall survival for patients with MIBC or NMIBC.

SNP/genotype	MIBC			NMIBC		
	Alive/dead	HR (95% CI)[a]	*P* value[a]	Alive/dead	HR (95% CI)[a]	*P* value[a]
rs12921862						
CC	75/22			96/12		
CA	34/13			58/1		
AA	2/3			0/0		
Codominant		1.41 (0.83-2.42)	0.21		0.10 (0.01-0.84)	***0.03***
Dominant		1.45 (0.74-2.88)	0.28		0.10 (0.01-0.84)	***0.03***
Recessive		1.94 (0.53-6.89)	0.32		NA	NA
rs1805105						
TT	54/19			89/6		
TC	46/16			56/7		
CC	11/2			8/0		
Codominant		1.40 (0.71-2.76)	0.34		0.97 (0.34-2.72)	0.95
Dominant		1.21 (0.58-2.54)	0.62		1.12 (0.35-3.62)	0.85
Recessive		4.28 (0.88-20.88)	0.07		0.00 (0.00-NA)	0.99
rs370681						
CC	37/10			60/7		
CT	55/23			69/5		
TT	16/3			16/0		
Codominant		1.30 (0.72-2.34)	0.38		0.54 (0.19-1.54)	0.25
Dominant		1.76 (0.76-4.06)	0.18		0.60 (0.18-1.97)	0.40
Recessive		0.82 (0.21-3.17)	0.77		0.00 (0.00-NA)	0.99

Italic values indicate a significant difference at the 5% level. [a]Adjusted by age, sex, smoking status, tumor stage, tumor grade, recurrence status, and metastasis status. HR: hazard ratio; CI: confidence interval.

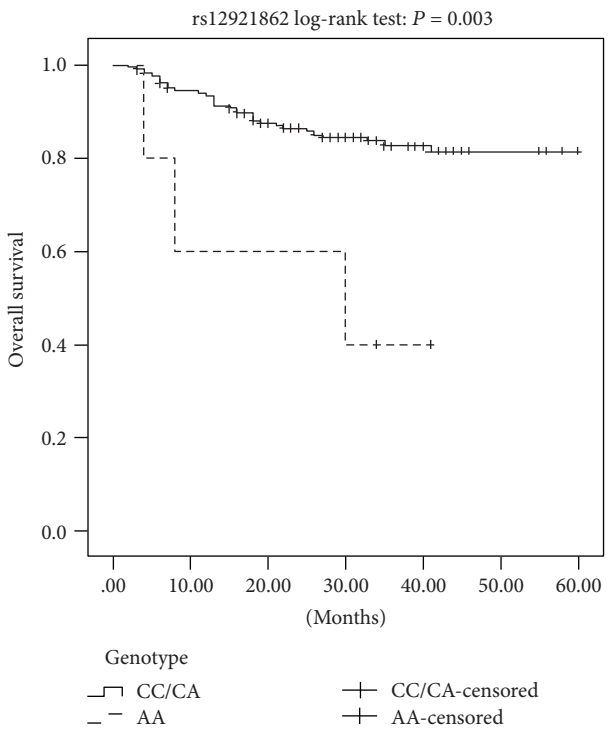

Figure 1: Kaplan-Meier survival curves for the recessive model of *AXIN1* rs12921862 polymorphism in BC patients.

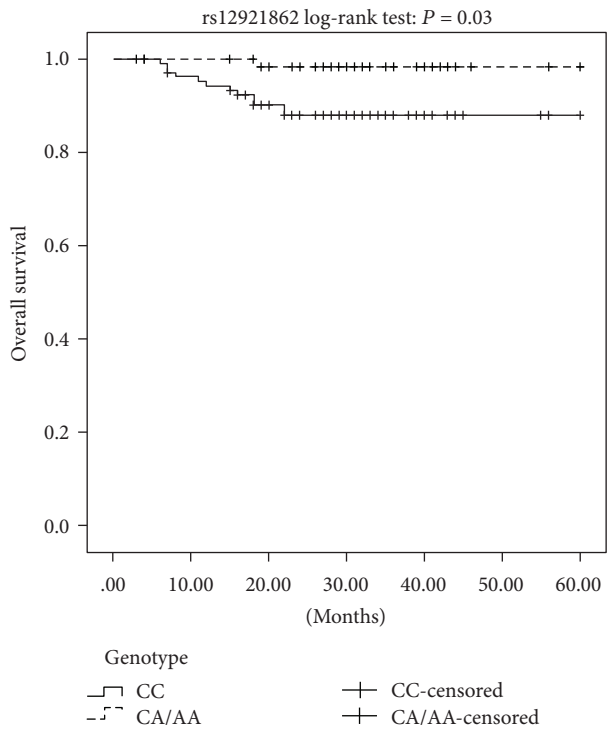

Figure 2: Kaplan-Meier survival curves for the dominant model of *AXIN1* rs12921862 polymorphism in NMIBC patients.

rs12921862 SNP had significantly higher frequencies of patients older than 64 years compared to those under 64 years, indicating that the A allele could be one of the risk factors of increased BC susceptibility in the older population.

A correlation between reduced *AXIN1* expression and tumor progression has been reported in esophageal squamous cell carcinoma, and several mutations and polymorphisms of *AXIN1* have been found in squamous cell tumors and cell lines [23, 34, 35]. Several recent studies have detected *AXIN1* gene sequence variations in subsets of ovarian endometrial adenocarcinomas and advanced prostate cancer, indicating new potentially relevant *AXIN1* mutations [23, 36]. Furthermore, Chimge et al. found that AXIN1 could be a potential target for the management of ER^+ breast cancer [14]. Our study demonstrated the association of certain *AXIN1* gene polymorphisms with higher BC susceptibility and higher overall survival rate of NMIBC patients. Our results provide a new insight into potential predictive factors of BC progression and prognosis.

5. Conclusions

In conclusion, *AXIN1* SNPs are potential risk factors of BC susceptibility, rs370681 is associated with a severe form of BC, and rs12921862 is a significant forecast factor to BC prognosis. However, our study has several limitations. We did not analyze the expression levels of *AXIN1* in the patients' sera and tumor tissues. Also, we only studied a cohort of southwest China, whereas different ethnic populations differ in the types and frequencies of genetic polymorphisms. Therefore, further studies on larger and more diverse cohorts are needed to validate *AXIN1* SNPs as reliable markers to predict the progression and prognosis of bladder cancer.

Data Availability

The data used to support the findings of this study are currently under embargo while the research findings are commercialized. Requests for data, 6 months after publication of this article, will be considered by the corresponding authors.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Qin Li and Peng Zhang contributed equally to this work.

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Research Article

High SALM3 Expression in Tumor Cells and Fibroblasts Is Correlated with Poor Prognosis in Gastric Cancer Patients

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Objective. The synaptic adhesion-like molecule (SALM) family is largely restricted to neural tissues and is involved in the regulation of neurite outgrowth and synapse formation. However, the expression of SALM3 in gastric cancer (GC) and its clinical significance remain unclear. The aim of the present study was to investigate the prognostic value of SALM3 in patients with GC. *Patients and Methods*. Expression of SALM3 was validated by tissue microarrays from 730 GC patients and statistically assessed for correlations with the clinical parameters and the prognosis of the patients. The transcriptional and survival data of SALM3 in GC patients were also mined through the Oncomine and Kaplan-Meier Plotter databases. *Results*. SALM3 is overexpressed in the tumor cells and fibroblasts of clinical GC tissues, and a high level of SALM3 was significantly associated with tumor invasive characteristics. Cox proportional hazards univariate and multivariate regression analyses revealed SALM3 expression in tumor cells or stroma as an independent prognostic factor in the overall survival rate of GC patients. Furthermore, the survival of GC patients with high SALM3 expression in both tumor cells and fibroblasts was significantly poorer than that of the other groups. Oncomine and Kaplan-Meier Plotter analyses further confirmed high levels of SALM3 expression in GC, and high levels of SALM3 expression were associated with shorter survival in patients. *Conclusion*. SALM3 may be a prognostic factor for GC and may potentially be a high-priority therapeutic target.

1. Introduction

Gastric cancer (GC) is the second leading cause of cancer-associated mortality worldwide, and it is the most common gastrointestinal malignancy in Eastern Europe and East Asia, especially in China [1–3]. Although considerable advances have been achieved in early diagnosis, surgical techniques, and medical treatment, more than half of patients at the advanced stage of the disease die of cancer recurrence and metastasis, even after receiving radical gastrectomy [4]. Post-operative recurrence and metastasis are the biggest obstacles to the treatment of GC [5]. In view of the high frequency of new cases and the adverse outcomes in GC, there has been an exploration for biologic markers that are associated with the development and prognosis of this disease. Nevertheless, to date, no such markers have been found as ideal clinical predictive factors for the diagnosis, therapy, or prognosis of GC. Therefore, it is essential to identify novel prognostic and predictive markers, which will aid in novel effective therapies for GC.

The synaptic adhesion-like molecule (SALM) family of adhesion molecules, also known as Leucine-rich repeat and fibronectin type III domain-containing (LRFN), belongs to the superfamily of leucine-rich repeat-containing adhesion molecules [6, 7]. Previous reports showed that the protein expression of the SALM family, which includes five known members, is largely restricted to neural tissue, and these proteins play vital roles in neuritis growth and branching and synapse formation and maturation [8, 9]. However, recently, some research has demonstrated that SALM family members are also expressed and function in nonneural tissues. Some studies showed that SALM1/LRFN2 was involved

in erythropoiesis [10] and that SALM2/LRFN1 participates in pancreatic cancer cell survival [11]. Furthermore, Konakahara et al. [12] demonstrated that SALM3/LRFN4 expression in monocytic cell line THP-1 was upregulated with macrophage differentiation. Additionally, SALM3/LRFN4 signaling plays a vital role in monocyte/macrophage migration [13]. In addition, they also showed that SALM3/LRFN4 was expressed in a variety of human leukemia and cancer cell lines, such as Jurkat, MKN45, SW480, and PANC-1 [12]. Up to now, the expression pattern of SALM3 and its clinical significance in gastric cancer remains poorly understood.

Until recent years, the principal focus of cancer research has mostly been on the malignant cells themselves. In fact, the growth of a tumor is not determined only by cancer cells, because interactions between malignant cells and stromal compartments have a major impact on cancer growth and progression [14]. Advances in understanding the contribution of fibroblasts to cancer progression will enhance our awareness and knowledge about this reciprocal signaling, which supports and promotes the growth, dedifferentiation, invasion, and survival of tumors [15]. More and more studies have specifically showed the expression of tumor markers in fibroblasts and found them to be closely involved in cancer progression and patients' prognoses [16]. Kessenbrock et al. [17] speculated on the multiple functions of matrix metalloproteinases (MMPs) in the tumoral stroma and categorized these proteases according to roles in tissue angiogenesis, invasion, and intravasation, as well as in the preparation of the metastatic niche. A better understanding of the cross talk between the cancer cells and the fibroblasts will enhance our knowledge about the growth-promoting signaling pathways and finally lead to new therapeutic interventions targeting the tumor stroma [15].

In the present study, we evaluated the relationship between SALM3 expression in GC cells and tumoral stroma in tissue microarrays (TMAs) by immunohistochemistry (IHC) and clinicopathologic characteristics including prognostic significance. In addition, we explored the expression of SALM3 in gastric cancer *vs.* normal tissues based on the Oncomine databases, as well as its corresponding prognostic value in the Kaplan-Meier Plotter databases.

2. Materials and Methods

2.1. Patients and Specimens. From January 2004 to November 2009, 730 GC and 20 matched adjacent nontumor tissues were taken from radical surgical procedures; also, 27 chronic gastritis, 26 intestinal metaplasia, 32 low-grade intraepithelial neoplasia, and 25 high-grade intraepithelial neoplasia tissues were acquired through gastric endoscopic biopsies and were randomly obtained from the clinical biobank of the Affiliated Hospital of Nantong University. Of the samples, none of the 730 GC patients had received any type of treatment before surgery. All GC patients were observed until March 2017, with a median observation time of 42.5 months. Follow-up procedures were described in our previous studies [18]. At the last follow-up, 314 (43.01%) patients had died from either recurrence of the disease ($n = 274$) or surgery-related complications without recurrence ($n = 140$). Among the remaining 416 patients, the mean duration of follow-up was 72.5 months (range: 17.4-130.4 months, standard deviation: ±11.2). Overall survival (OS) was defined as the interval between the dates of surgery and death. Progression-free survival (PFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Human Research Ethics Committee of our hospital. Written informed consent was obtained from all study participants.

2.2. TMA Construction and Immunohistochemistry Analysis. TMAs were constructed as our previous reports [19]. Briefly, two cores from representative blocks of the formalin-fixed and paraffin-embedded tissues were used to construct TMA slides using the manual Quick Ray Tissue Microarrayer System (UT06, Unitma Co. Ltd., South Korea), which we have in the Department of Clinical Pathology of our hospital.

IHC was carried out using a rabbit polyclonal anti-human SALM3 antibody (1 : 80, MAB5445, R&D Systems, Minneapolis, MN, USA) with a EnVision+™ peroxidase kit (Dako, Carpinteria, CA, USA). Secondly, samples were incubated with 3,3′-diaminobenzidine (Dako, Carpinteria, CA, USA). Negative controls were performed identically but without the primary antibodies. SALM3 staining was semiquantitatively assessed using the H-score method [20] depending on the scores of staining intensity (0 as no staining, 1+ as weak staining, 2+ as moderate staining, and 3+ as intense staining) and the scores of percentages of positive tumor cells (0 as 0-20%, 1 as 21-50%, 2 as 51-70%, and 3 as 71-100%). The final IHC scores were defined as the product of staining intensity and percentages which was calculated as ranging from 0 to 300. All scores of cases were reviewed and calculated by two independent pathologists without any knowledge of the clinical characteristics.

2.3. Oncomine Analysis. To determine the SALM3 expression pattern in GC, we used the datasets in the Oncomine Cancer Microarray Database (https://www.oncomine.org) [21]. In order to analyze the messenger ribonucleic acid (mRNA) levels of SALM3 in GC, the mRNA expressions of SALM3 in clinical cancer specimens were compared with those in normal controls, with a Student t-test to generate a p value. The fold change and the cutoff of the p value were defined as 2 and 0.01, respectively.

2.4. The Kaplan-Meier Plotter. To analyze the prognostic value of the mRNA expression of SALM3, we used the Kaplan-Meier Plotter (http://www.kmplot.com) [22], which includes the gene expression data and survival information of 1,065 clinical gastric cancer patients [23]. Depending on the median expression of SALM3, we analyzed the PFS and OS of patients with GC who were divided into two cohorts with low and high expression, by the hazard ratio (HR) with log-rank p value and 95% confidence intervals (CI).

2.5. Statistical Analysis. All analyses were conducted with the SPSS 20.0 software (IBM Corporation, Armonk, NY, USA).

The X-Tile software (Rimm Lab, Yale University School of Medicine, New Haven, USA) was used to analyze the cutoff values for low or high SALM3 levels. Pearson's chi-square test was used to determine the correlation between the expression of SALM3 and clinicopathological parameters. The Kaplan-Meier method and the log-rank survival analysis were used to generate the survival curves. Univariate and multivariate analyses were performed with a Cox proportional hazards model to identify the prognostic factors. p values < 0.05 were considered statistically significant.

3. Results

3.1. SALM3 Protein Expression in GC by IHC. To investigate the SALM3 expression in clinical GC tissues, we performed IHC analysis with TMAs, which contained matched nontumor tissues and complete clinical outcome information. Positive staining of SALM3 was primarily localized in tumor cell cytoplasm and membranes and in fibroblasts. Representative IHC SALM3 staining patterns are shown in Figure 1(a). Furthermore, we used the X-Tile software to measure the cutoff values for high or low SALM3 expression. Here, 60 was determined as the cutoff point for SALM3 in tumor and stromal cells; scores from 0 to 60 were considered as low expression and scores from 61 to 300 were deemed as high expression. In cancer cells, high SALM3 protein levels were detected in 340 (46.6%) of 730 GC tissues and showed statistical significance ($\chi^2 = 62.87$, $p < 0.001$). In addition, a significantly high SALM3 expression in stromal cells was identified in 261 (35.8%) of 730 GC tissues ($\chi^2 = 59.39$, $p < 0.001$) (Table 1).

3.2. Database Analysis Reveals That SALM3 Is Upregulated in GC. To determine the clinical significance of SALM3 in patients with GC, we performed data mining and analyzed SALM3 mRNA levels from the publicly available Oncomine database. The finding for SALM3 mRNA expressions based on 6 databases identified 3 with a significant p value ($p < 0.001$), and this gene ranks in the top 10% among all differentially expressed genes. We collected the results from Cho's [24], DErrico's [25], and Wang's [26] studies and analyzed SALM3 mRNA expression in GC. SALM3 expression was found higher in gastric normal mucosa than in cancer, even when stratified into intestinal-, diffuse-, and mixed-type carcinomas by Lauren's classification ($p < 0.05$, Figure 1(b)).

The median rank of SALM3 in upregulated genes of GC was 210.0 based on a meta-analysis across the three above datasets, including 7 analyses using Oncomine algorithms [27] ($90 + 69 + 27 + 502$ samples, $p = 5.72E - 5$, Figure 1(c)).

3.3. Expression Level of SALM3 in Tumor Cells and Fibroblasts and GC Patients' Survival. Among the 730 GC patients, the Kaplan-Meier survival analysis indicated that the high expression of SALM3 in tumor cells ($p < 0.001$) or stroma ($p < 0.001$) was significantly associated with poor OS (Figures 2(a) and 2(b)). The multivariate analysis demonstrated that in addition to conventional clinicopathological parameters, such as nodal status and metastasis, SALM3 in tumor cells ($p < 0.001$) and fibroblasts ($p < 0.001$) was an independent unfavorable factor for OS (Table 2). To evaluate the combined effect of SALM3 on the prognosis of GC, we classified patients into four subgroups according to the SALM3 expression in tumor cells and fibroblasts: group I had low expression of the two distributions, group II had low tumor cell and high fibroblast expression, group III had high tumor cell and low fibroblast expression, and group IV had a high expression of both distributions. We found that the OS of group IV was significantly lower than that of the other groups ($p = 0.023$, Figure 2(c)).

We also used the Kaplan-Meier Plotter to analyze the prognostic significance of SALM3 mRNA. Based on the data from the Kaplan-Meier Plotter, SALM3 mRNA expression was positively correlated to both OS and PFS rates of the patients with GC (Figure 2(d), $p < 0.05$).

3.4. Upregulation of SALM3 Is Correlated with Advanced Clinicopathological Features of GC. We analyzed the correlation between SALM3 expression and the clinicopathological characteristics of GC. Strong associations were observed between SALM3 expression in tumor cells and tumor classification ($p < 0.001$), lymph node metastasis ($p = 0.019$), tumor metastasis ($p = 0.004$), and TNM stage ($p < 0.001$) (Table 3). High SALM3 expression in fibroblasts was significantly associated with tumor classification ($p < 0.001$), lymph node metastasis ($p < 0.001$), and TMN stage ($p < 0.001$) (Table 3). These results significantly indicated a correlation between the expression of SALM3 and an unfavorable prognosis of GC.

4. Discussion

In the present study, we revealed that SALM3 was highly expressed in GC and fibroblasts and was significantly associated with clinical parameters and reduced survival time of patients with GC. Multivariate analysis showed that SALM3 expression in GC cells and fibroblasts might be an independent prognostic factor of survival in patients with GC.

Some research has previously demonstrated that the expression and function of the members of the SALM family are mostly restricted to neural tissues [28]. Nevertheless, a recent study reported that SALM3, also known as LRFN4, is expressed in some cancer cell lines, such as Panc-1, JURKAT-1, and MKN7 [12]. In the present study, we found that protein expression of SALM3 was primarily localized in the gastric cancer cell cytoplasm. We also found that SALM3 protein expression in cancer samples was higher than that in paracancer tissues and benign gastric disease tissues. Moreover, high SALM3 expression in GC was associated with certain clinicopathological characteristics, such as primary cancer, distant metastasis, and TNM stage. Our results showed that SALM3 plays a protumorigenic role in gastric cancer. Consistent with this conclusion, we found that the upregulation of SALM3 is generally correlated with an adverse prognosis of gastric cancer patients. Oncomine data expression analysis also showed that SALM3 is upregulated in gastric adenocarcinoma tumor, which provides another layer of evidence that SALM3 might positively take part in the regulation and development of GC.

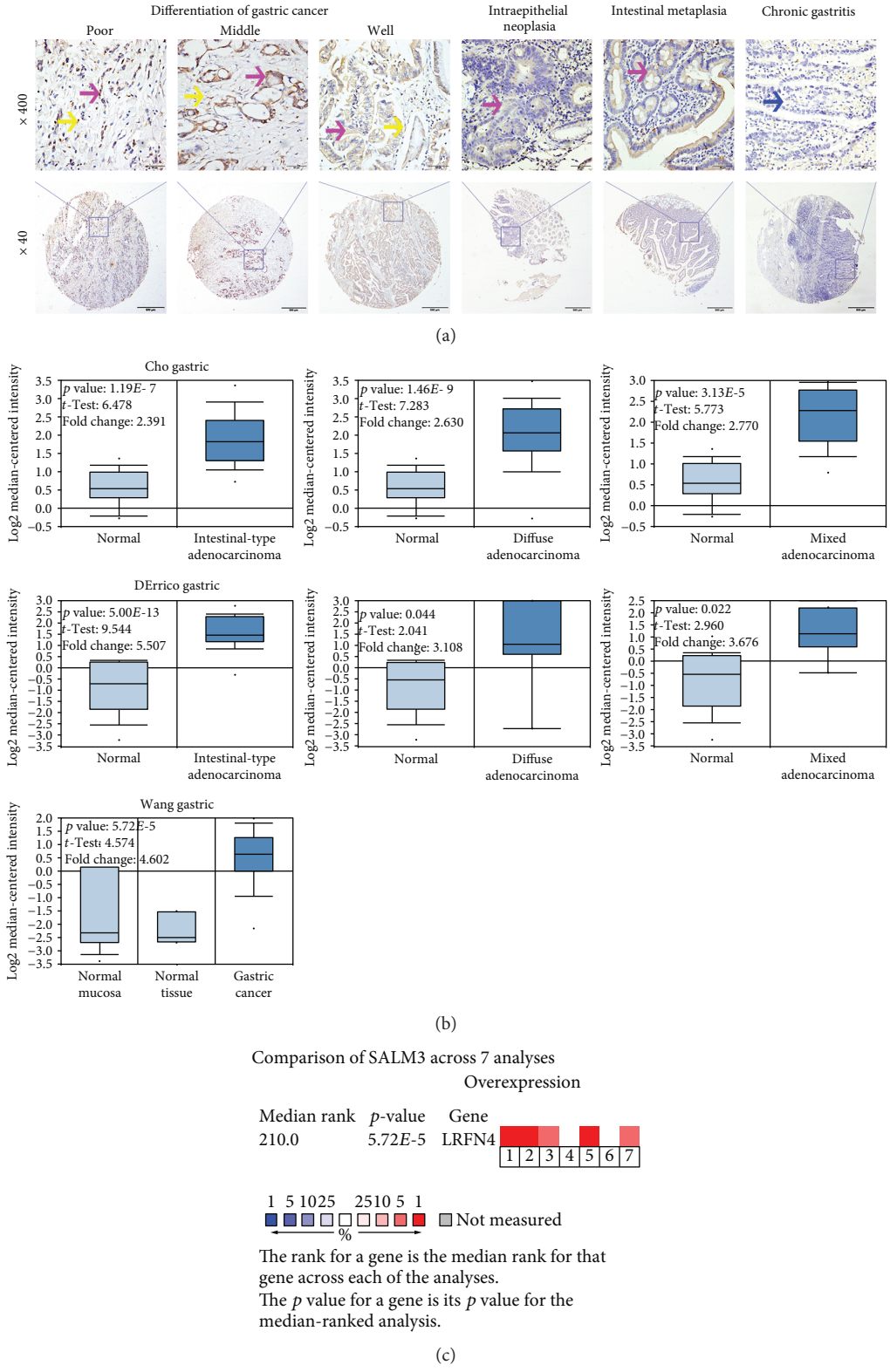

Figure 1: High expression of SALM3 in gastric cancer cells and fibroblasts. (a) Representative images of SALM3 expression in benign and malignant gastric tissue samples: positive tumor cytoplasm (purple arrow), fibroblasts (yellow arrow), and negative staining (blue arrow) of immunohistochemical staining of SALM3. Rows 1 and 2 are SALM3 staining at a magnification of 400x (bar = 50 μm) and 40x (bar = 500 μm), respectively. (b) Box plots from gene expression data in Oncomine comparing the expression of SALM3 in normal and GC tissues. The p value was set up at 0.01, and fold change was defined as 2. (c) A meta-analysis of SALM3 gene expression from 3 Oncomine databases, where colored squares indicate the median rank for SALM3 (vs. normal tissue) across 7 analyses: Cho's gastric (1-3), DErrico's gastric (4-6), and Wang's gastric (7). The p value is given for the median-rank analysis.

Table 1: SALM3 expression in gastric benign and malignant tissues.

Characteristic	n	SALM3 in tumor cells				SALM3 in fibroblasts			
		SALM3- (%)	SALM3+ (%)	Pearson χ^2	p	SALM3- (%)	SALM3+ (%)	Pearson χ^2	p
Total	860	511 (59.4)	349 (40.6)	62.87	<0.001	596 (69.3)	264 (30.7)	59.39	<0.001
Chronic gastritis	27	26 (96.3)	1 (3.7)			27 (100.0)	0 (0.0)		
Intestinal metaplasia	26	25 (96.2)	1 (3.8)			26 (100.0)	0 (0.0)		
Low-grade intraepithelial neoplasia	32	29 (90.6)	3 (9.4)			32 (100.0)	0 (0.0)		
High-grade intraepithelial neoplasia	25	22 (88.0)	3 (12.0)			22 (88.0)	3 (12.0)		
Matched tumor neighbor	20	19 (95.0)	1 (5.0)			20 (100.0)	0 (0.0)		
Cancer	730	390 (53.4)	340 (46.6)			469 (64.2)	261 (35.8)		

*$p < 0.05$; SALM3- represents low expression and SALM3+ represents high expression.

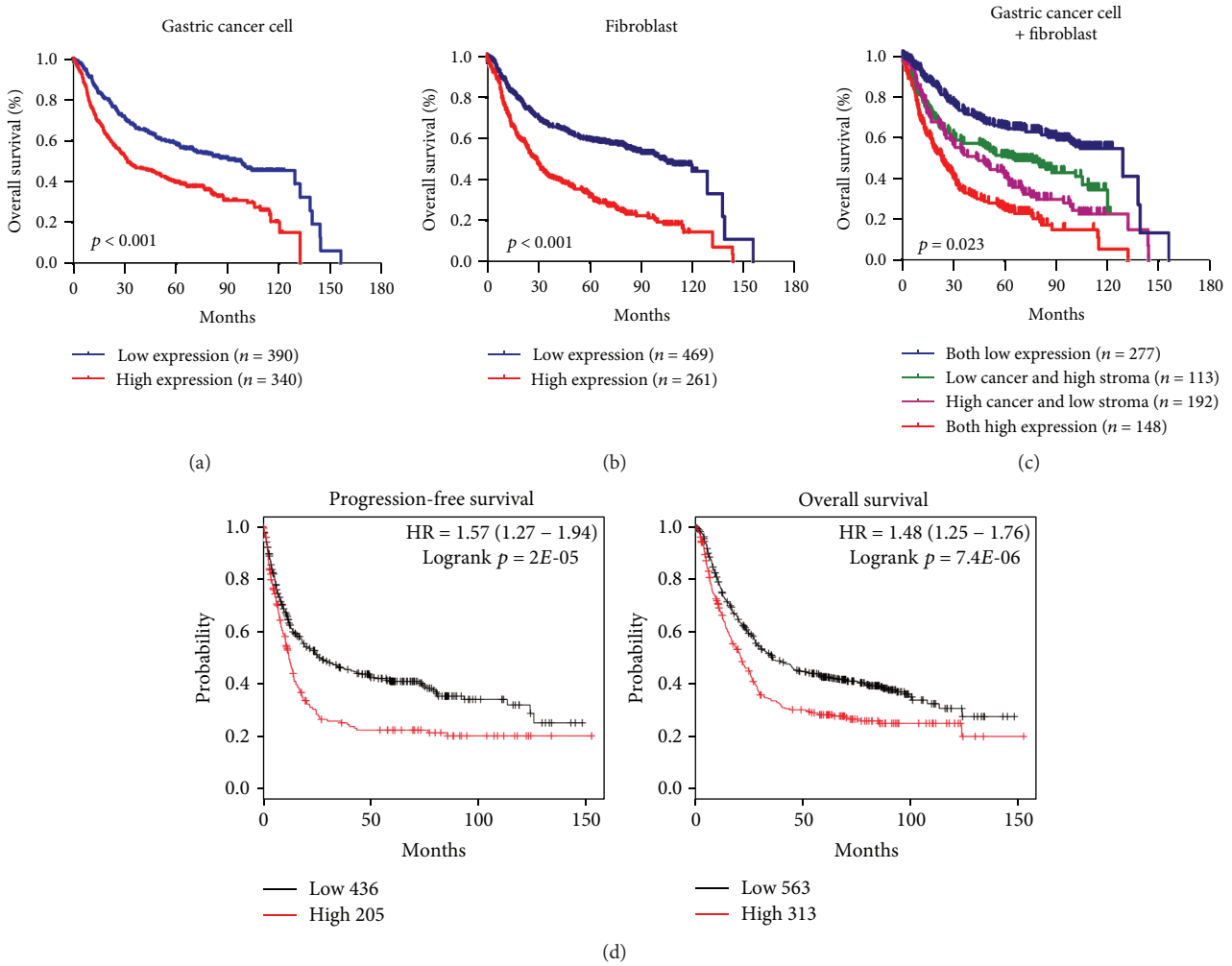

Figure 2: The prognostic values of SALM3 in gastric cancer. High levels of SALM3 in tumor cells (a), stroma (b), and four groups of tumor cells and stroma (c) were associated with shorter overall survival in GC patients of the Nantong cohort. Four groups were classified according to the SALM3 expression in tumor cells and fibroblasts. (d) Based on the data from Kaplan-Meier Plotter, high SALM3 mRNA levels were positively related to both progression-free survival and overall survival of patients with GC.

The cross talk between the cancer cells and tumoral stroma is significantly associated with the progression and metastasis of tumor [29]. During recent years, many studies have specifically identified and demonstrated some tumor markers expressed in the tumor stroma that are closely correlated with tumor progression and patients' adverse

Table 2: Univariate and multivariate analysis of prognostic factors for overall survival in gastric cancer patients.

	Univariate analysis			Multivariate analysis		
	HR	$p > \|z\|$	95% CI	HR	$p > \|z\|$	95% CI
Gender						
Male vs. female	1.014	0.903	(0.816, 1.258)			
Age						
≤60 vs. >60	1.167	0.116	(0.963, 1.413)			
Histological type						
Tubular vs. mucinous vs. mixed (tubular and mucinous) vs. signet ring cells vs. others[a]	1.018	0.698	(0.930, 1.114)			
Differentiation						
Well vs. middle vs. poor	1.526	<0.001*	(1.273, 1.829)	1.096	0.366	(0.898, 1.339)
T						
Tis vs. T1+T2 vs. T3+T4	2.723	<0.001*	(2.207, 3.360)	1.317	0.083	(0.964, 1.798)
N						
N0 vs. N1 vs. N2 vs. N3	1.700	<0.001*	(1.568, 1.844)	1.265	0.002*	(1.093, 1.463)
M						
M0 vs. M1	3.503	<0.001*	(2.556, 4.800)	2.176	<0.001*	(1.534, 3.088)
TNM stage						
0+I vs. II vs. III+IV	2.686	<0.001*	(2.331, 3.096)	1.453	0.022*	(1.054, 2.004)
SALM3 expression in tumor cells						
High vs. low or none	1.785	<0.001*	(1.473, 2.165)	1.471	<0.001*	(1.210, 1.789)
SALM3 expression in stroma						
High vs. low or none	2.262	<0.001*	(1.868, 2.740)	1.525	<0.001*	(1.247, 1.865)
Combination of tumor cells and fibroblasts						
I vs. IV	2.137	0.023*	(1.124, 2.537)	1.621	0.036*	(1.312, 2.472)
II vs. IV	1.276	0.616	(0.714, 2.328)			
III vs. IV	1.769	0.548	(1.357, 2.664)			

*$p < 0.05$; [a]Others: papillary adenocarcinoma, 5 cases; adenosquamous carcinoma, 6 cases; squamous cell carcinoma, 7 cases; undifferentiated carcinoma, 1 case; small cell malignant tumor, 9 cases; carcinoid, 2 cases; focal cancer, 2 cases. Four groups were classified according to the SALM3 expression in tumor cells and/or stroma: group I has low expression of both, group II has high expression of tumor cells and low expression of stroma, group III has low expression of tumor cells and high expression of stroma, and group IV has high expression of both.

prognoses. In the present study, we found that high SALM3 expression in the fibroblasts of gastric cancer tissues was related with regional lymph node metastasis and advanced TNM stage, and it independently predicted unfavorable OS for cancer patients. Furthermore, in terms of SALM3 expression in cancer cells and stroma, GC patients with cancer-cells^{high} and fibroblasts^{high} had worse prognosis than the other groups. Cancer is composed of not only simply autonomous malignant cells but fibroblasts, endothelial cells, immune cells, and specialized mesenchymal cells. The neoplastic cells can recruit these different types of stroma cells to facilitate the growth of the tumor and contribute to distant metastasis [30]. Importantly, tumor-infiltrating immune cells, especially the presence of macrophages at the margins of tumors, have been noted to be significantly related to the stimulation of cancer cell proliferation, tissue invasion, and support of cancer cell seeding and further metastatic dissemination, via inducing and helping sustain tumor angiogenesis [23, 31]. Interestingly, it was reported that SALM3 expression is upregulated in monocytic cells with macrophage differentiation [12]. Additionally, SALM3 signaling plays a vital role in inducing the migration of monocytes/macrophages into the inflammation area [12, 13]. Therefore, we speculate that SALM3 might participate in the procedure of dissemination and recurrence of gastric cancer.

Nevertheless, there are several limitations in our research. We need to apply further larger prospective studies to the general population for confirmation to correct the shortcomings of a retrospective observational study. Additionally, as a semiquantitative IHC data study, it needs additional methods to evaluate and confirm SALM3 expression in tumor cells and stroma. Furthermore, we should investigate the mechanisms of SALM3 in tumorigenesis by *in vitro* studies in the next step.

5. Conclusions

We found that SALM3 is upregulated in gastric cancer tissues and SALM3 expression is negatively correlated with patients' survival. In a word, it is suggested that SALM3 can serve as a potential marker for predicting clinical prognosis and a therapeutic target for gastric cancer patients.

Table 3: Association of SALM3 expression with clinicopathological characteristics in gastric cancer patients.

Characteristic	n	SALM3 in tumor cells				SALM3 in fibroblasts			
		SALM3- (%)	SALM3+ (%)	Pearson χ^2	p	SALM3- (%)	SALM3+ (%)	Pearson χ^2	p
Total	730								
Age				0.233	0.629			1.054	0.305
>60	387	210 (54.3)	177 (45.7)			242 (62.5)	145 (37.5)		
≤60	343	180 (52.5)	163 (47.5)			227 (66.2)	116 (33.8)		
Gender				0.236	0.627			0.031	0.860
Male	537	284 (52.9)	253 (47.1)			344 (64.1)	193 (35.9)		
Female	193	106 (54.9)	87 (45.1)			125 (64.8)	68 (35.2)		
Histological type				5.428	0.246			9.273	0.055
Tubular	614	328 (53.4)	286 (46.6)			403 (65.6)	211 (34.4)		
Mucinous	31	21 (67.7)	10 (32.3)			14 (45.2)	17 (54.8)		
Mixed (tubular and mucinous)	16	7 (43.8)	9 (56.3)			7 (43.8)	9 (56.3)		
Signet ring cell	37	21 (56.8)	16 (43.2)			26 (70.3)	11 (29.7)		
Others[a]	32	13 (40.6)	19 (59.4)			19 (59.4)	13 (40.6)		
Differentiation				5.008	0.082			4.685	0.096
Well	38	27 (71.1)	11 (28.9)			28 (73.7)	10 (26.3)		
Middle	193	101 (52.3)	92 (47.7)			133 (68.9)	60 (31.1)		
Poor	499	262 (52.5)	237 (47.5)			308 (61.7)	191 (38.3)		
T				18.699	<0.001*			29.759	<0.001*
Tis	41	30 (73.2)	11 (26.8)			36 (87.8)	5 (12.2)		
T1+T2	220	136 (61.8)	84 (38.2)			164 (74.5)	56 (25.5)		
T3+T4	469	224 (47.8)	245 (52.2)			269 (57.4)	200 (42.6)		
N				9.958	0.019*			73.214	<0.001*
N0	303	178 (58.7)	125 (41.3)			238 (78.5)	65 (21.5)		
N1	135	72 (53.3)	63 (46.7)			97 (71.9)	38 (28.1)		
N2	139	74 (53.2)	65 (46.8)			64 (46.0)	75 (54.0)		
N3	153	66 (43.1)	87 (56.9)			70 (45.8)	83 (54.2)		
M				8.139	0.004*			2.453	0.117
M0	680	373 (54.9)	307 (45.1)			442 (65.0)	238 (35.0)		
M1	50	17 (34.0)	33 (66.0)			27 (54.0)	23 (46.0)		
TNM stage				19.053	<0.001*			72.728	<0.001*
0+I	184	120 (65.2)	64 (34.8)			149 (81.0)	35 (19.0)		
II	250	137 (54.8)	113 (45.2)			183 (73.2)	67 (26.8)		
III+IV	296	133 (44.9)	163 (55.1)			137 (46.3)	159 (53.7)		

*$p < 0.05$; [a]Others: papillary adenocarcinoma, 5 cases; adenosquamous carcinoma, 6 cases; squamous cell carcinoma, 7 cases; undifferentiated carcinoma, 1 case; small cell malignant tumor, 9 cases; carcinoid, 2 cases; focal cancer, 2 cases.

Data Availability

The data of mRNA levels of SALM3 used to support this study are available at the datasets in the Oncomine Cancer Microarray Database (https://www.oncomine.org). The prognostic data of the mRNA expression of SALM3 supporting this study are from Kaplan-Meier Plotter (http://www.kmplot.com). The IHC data used to support the findings of this study are included within the article.

Conflicts of Interest

There are no conflicts of interest.

Authors' Contributions

Ying Liu and Xiaoli Chen contributed equally to this study.

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Research Article

High Expression of LTBP2 Contributes to Poor Prognosis in Colorectal Cancer Patients and Correlates with the Mesenchymal Colorectal Cancer Subtype

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Colorectal cancer (CRC) is a complex and heterogeneous disease with four consensus molecular subtypes (CMS1-4). LTBP2 is a member of the fibrillin/LTBP super family and plays a critical role in tumorigenesis by activating TGF-β in the CMS4 CRC subtype. So far, the expression and prognostic significance of LTBP2 in CRC remains obscure. In this study, we aimed to analyze the mRNA and protein expression levels of LTBP2 in CRC tissues and then estimate their values as a potential prognostic biomarker. We detected the mRNA expression of LTBP2 in 28 cases of fresh CRC tissues and 4 CRC cell lines and the protein expression of LTBP2 in 483 samples of CRC tissues, matched tumor-adjacent tissues, and benign colorectal diseases. LTBP2 protein expression was then correlated to patients' clinical features and overall survival. Both LTBP2 mRNA and protein expression levels in CRC tissues were remarkably superior to those in adjacent normal colorectal tissues ($P = 0.0071$ and $P < 0.001$, respectively), according to TCGA dataset of CRC. High LTBP2 protein expression was correlated with TNM stage ($P < 0.001$), T stage ($P < 0.001$), N stage ($P < 0.001$), and M stage ($P < 0.001$). High LTBP2 protein expression was related to poor overall survival in CRC patients and was an independent prognostic factor for CRC. LTBP2 mRNA expression was especially higher in the CMS4 subtype ($P < 0.001$), which was confirmed in CRC cell lines. Our data suggested that LTBP2 may act as an oncogene in the development of colorectal cancer and have important significance in predicting CRC prognosis. LTBP2 could be a novel biomarker and potential therapeutic target for mesenchymal colorectal cancer and can improve the outcome of high-risk CRC.

1. Introduction

Colorectal cancer (CRC) demonstrates extremely heterogeneous and complex gastrointestinal malignancy, with increasing incidence and mortality according to the newest statistical survey [1]. Despite the remarkable advances in the management of CRC in recent years, the 5-year overall survival (OS) rate is still poor [2, 3]. General thinking suggests that genetic alterations, including somatic gene mutation, deletion, or amplification, copy number variation, and epigenetic modifications, facilitate initiation and progression of CRC. Through the integrated analysis of transcriptomic data, CRC was divided into four subtypes named consensus molecular subtypes (CMS), with the following distinguishing molecular features: CMS1 (MSI immune, 14%); CMS2 (canonical, 37%); CMS3 (metabolic, 13%); and CMS4 (mesenchymal, 23%) [4–6]. Of these, CMS4 is closely related to recurrence and metastasis, and the underlying mechanisms include transforming growth factor β (TGF-β) activation, stromal invasion, and angiogenesis. It is usually at a relatively later tumor stage when it is diagnosed, and it contributes to worse relapse-free and overall survival rate [7]. Thus, CMS4

has been considered as the worst type of CRC outcome. Early diagnosis and subtype identification can significantly increase the OS rate of CRC; thus, it is urgent for us to find a novel and reliable biomarker for CRC which can improve the outcome of patients with CRC.

Latent transforming growth factor β binding protein 2 (LTBP2), a secreted extracellular matrix (ECM) protein, is a member of the fibrillin/LTBP super family, which contains LTBPs 1-4 and fibrillins 1, 2, and 3 [8, 9]. These proteins consist mainly of cysteine-rich EGF-like and 8-cysteine (8-Cys) repeats and share a similar overall domain structure. LTBPs 1, 3, and 4 regulate the biological activities of TGF-β family growth factors by covalently binding TGF-β and directing the growth factor to storage depots within the extracellular matrix, while LTBP2 is hypothesized to indirectly regulate the activation of TGF-β by competing with LTBP1 for the same binding site to fibrillin 1 in microfibrils [10, 11]. Accumulating evidence shows that LTBPs play important roles in tumorigenesis, especially LTBP2. A recent report demonstrated that LTBP2 was involved in the signaling pathway of the mesenchymal subtype in colorectal cancer [12]. However, the clinical implication of LTBP2 expression in CRC remains unknown.

In this study, we determined both the mRNA and protein expression levels of LTBP2 in CRC tissues and matched tumor-adjacent tissues by quantitative real-time polymerase chain reaction (qRT-PCR) and tissue microarray immunohistochemistry (TMA-IHC) analyses, respectively. Then, we used TCGA database and CRC cell lines to confirm our results. Finally, we correlated LTBP2 protein expression to patients' clinical characteristics and estimated its potential prognostic significance.

2. Materials and Methods

2.1. Tissue Samples and Patients' Clinical Information. A total of 511 patients were included in the study. They provided 56 fresh surgical samples, including 28 cancer tissues and 28 matched adjacent tissues, and 483 archived formalin-fixed paraffin-embedded (FFPE) tissue blocks, including 204 cancer tissues, 190 matched normal surgical margins, 23 chronic colitis tissues, 44 low-grade intraepithelial neoplasia (LIN) tissues, and 22 high-grade intraepithelial neoplasia (HIN) tissues. The 56 fresh surgical samples were received from the Affiliated Hospital of Nantong University between 2016 and 2017. The 483 FFPE tissue blocks were obtained between 2004 and 2014 and were used to construct the TMA using the Tissue Microarray System (Quick-Ray, UT06, Unitma Co. Ltd., Korea). All clinical features containing gender, age, tumor location, differentiation grade, TNM stage, local invasion, regional lymph node metastasis, distant metastasis, and preoperative serum carcinoembryonic antigen (CEA) level were obtained from patients' medical records. All of these patients did not receive any therapy, such as radiotherapy, chemotherapy, and immunotherapy, prior to surgery. Patients were followed up for more than 5 years. This study was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University.

2.2. Cell Lines and Cell Culture. Human CRC cell lines (DLD1 and HT29 non-CMS4 subtypes and SW620 and Caco2 CMS4 subtypes) and a normal colorectal epithelial cell line (NCM460) were purchased from the Chinese Academy of Sciences (Shanghai, China). HT29, Caco2, DLD1, SW620, and NCM460 were maintained in McCoy's 5A medium (Gibco, Grand Island, NY, USA), RPMI 1640 medium (Corning, VA, USA), and DMEM medium (Corning, VA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA) and 1% Penicillin/Streptomycin (Gibco, BRL), respectively. All of them were cultured in 5% CO_2 atmosphere at 37°C. All media were changed every 2-3 days until 90% confluent, and cultures were split using 0.25% trypsin (Gibco, Canada).

2.3. RNA Extraction and qRT-PCR Analysis. Total RNA was isolated from CRC tissues and cell lines using the TRIzol Reagent (Invitrogen, CA, USA), and the cDNA was generated using SuperScript® III Reverse Transcriptase (Invitrogen, CA, USA). LTBP2 mRNA levels were measured by qRT-PCR. Relative quantification of mRNA was performed using the $\Delta\Delta$Ct method by first normalizing to the housekeeping gene GAPDH mRNA level and then normalizing to the reference sample. The reverse transcription conditions were 60 min at 42°C and 5 min at 72°C, and the conditions were saved at 4°C. For qRT-PCR, the conditions were 5 min at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 32 s, and 72°C for 30 s. The primers used are as follows: LTBP2 forward primer (5′-TTACAAGCAGAGACTCACT-3′) and LTBP2 reverse primer (5′-ACAACAGAAGAGACCAGAT-3′) and GAPDH forward primer (5′-GGACCAATACGAC CAAATCCG-3′) and GAPDH reverse primer (5′-AGCC ACATCGCTCAGACAC-3′).

2.4. Immunohistochemistry. LTBP2 protein expression in 483 tissue blocks was examined using IHC performed following the standard protocol [13]. After antigen retrieval, LTBP2 was detected by a rabbit polyclonal anti-human LTBP2 antibody (dilution 1 : 800, ab121193, Abcam, USA) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Abcam). The color was developed using 3,3′-diaminobenzidine (Dako, Carpinteria, CA, USA), counterstaining with hematoxylin. The LTBP2 protein expression level was quantified by a two-level grading system using an Olympus BX53 microscope (Olympus Co., Tokyo, Japan). LTBP2 staining intensity was scored as follows: 0 (−, no staining), 1 (+, mild staining), 2 (++, medium staining), or 3 (+++, intense staining). The percentage of positively stained cells (0-100%) was multiplied by the intensity score to give the final IHC score, which ranged from a minimum of 0 to a maximum of 300. The X-tile software program (Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab) was used to determine the cutoff value for low/high protein expression of LTBP2.

2.5. Statistical Analysis. SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The Pearson χ^2 test was applied to evaluate the relationships between the protein expression of LTBP2 and the patients' clinical

features. Kaplan-Meier survival curves and the log rank test were used for survival analysis and survival curve drawing. Univariate and multivariate Cox regression analyses were applied to assess the potential prognostic value of LTBP2 protein expression in CRC. P values less than 0.05 were considered to be statistically significant.

2.6. Bioinformatic Analysis of TCGA Database. The preprocessed level 3 RNA-seq data of colorectal cancer patients were collected from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov).

3. Results

3.1. LTBP2 mRNA Expression in CRC Tissues. We detected LTBP2 mRNA expression in 28 fresh CRC tissues and 28 matched adjacent tissues by qRT-PCR. LTBP2 mRNA expression was significantly higher in CRC tissues than in matched adjacent tissues ($P = 0.0071$, Figure 1(a)), in accord with TCGA database ($P < 0.0001$, Figure 1(b)).

3.2. LTBP2 Protein Expression in CRC Tissues. We determined LTBP2 protein expression in 483 archived tissue blocks including 204 cancer tissues, 190 matched normal surgical margins, 23 chronic colitis tissues, 44 low-grade intraepithelial neoplasia tissues, and 22 high-grade intraepithelial neoplasia tissues. The positive staining of LTBP2 was mainly localized in the cytoplasm of tumor cells (Figure 2). The X-tile software program was used to select the optimal cutoff value (180) for the low/high protein expression of LTBP2, which means that the scores from 0 to 180 and from 181 to 300 were regarded as low and high expressions, respectively. High LTBP2 protein expression was detected in 28.4% of CRC tissues, which was significantly higher compared with the expression detected in 4.3% of chronic colitis tissues, in 6.8% of low-grade intraepithelial neoplasia tissues, in 22.7% of high-grade intraepithelial neoplasia tissues, and in 6.8% of the surgical margin (Pearson $\chi^2 = 39.896$, $P < 0.001$) (Table 1).

3.3. Association between LTBP2 Protein Expression and Clinical Features in CRC Patients. The association between LTBP2 protein expression and clinical features in CRC patients is summarized in Table 2. CRC patients were divided into high-LTBP2 ($n = 58$) and low-LTBP2 ($n = 146$) groups according to the optimal cutoff value (180) of LTBP2 protein expression. High LTBP2 expression was obviously correlated with TNM stage ($\chi^2 = 38.118$, $P < 0.001$), T stage ($\chi^2 = 15.953$, $P < 0.001$), N stage ($\chi^2 = 20.443$, $P < 0.001$), and M stage ($\chi^2 = 24.24$, $P < 0.001$). Nevertheless, no significant correlations were observed between high LTBP2 protein expression and gender, age, tumor location, tumor differentiation, and preoperative CEA level.

3.4. Association between Survival, LTBP2 Protein Expression, and Clinical Features in CRC Patients. 204 CRC patients were followed up for a mean duration of 52.1 (range 0-100) months, and the 5-year overall survival (OS) rate was 55.9%. The Kaplan-Meier curve analysis showed that patients with high LTBP2 protein expression had an obviously shorter OS time than patients with low LTBP2 protein expression (log rank, $P < 0.0001$, Figure 3(a)), consistent with data from TCGA database ($P = 0.0316$, Figure 3(b)). Then, we analyzed the relationship between the OS rate and various prognostic factors in CRC patients using Cox regression univariate and multivariate analyses (Table 3). In the univariate analysis, LTBP2 protein expression (HR, 23.619, 95% CI: 13.036-42.794; $P < 0.001$), TNM stage (HR, 2.023, 95% CI: 1.532-2.672; $P < 0.001$), T stage (HR, 3.398, 95% CI: 1.692-6.825; $P < 0.001$), N stage (HR, 1.493, 95% CI: 1.207-1.847; $P < 0.001$), and M stage (HR, 5.983, 95% CI: 3.244-11.036; $P < 0.001$) were significantly correlated with OS. Afterwards, all the above factors were selected and put into the multivariate analysis to confirm whether LTBP2 protein expression was an independent factor of OS for CRC patients. Our results displayed that only LTBP2 protein expression (HR, 21.056, 95% CI: 11.274-39.326; $P < 0.001$) retained its significance and can be considered as an independent prognostic factor for OS of CRC patients (Table 3).

3.5. LTBP2 mRNA Expression in the CMS4 Subtype of CRC. Further analysis of TCGA database showed that LTBP2 mRNA expression was especially higher in the CMS4 subtype than in other subtypes in 450 CRC samples ($P < 0.0001$, Figure 4(a)). It was confirmed in CRC cell lines, which means that LTBP2 mRNA expression was significantly higher in Caco2 and SW620 which better represent the CMS4 CRC cell line compared with other subtype CRC cell lines (DLD1 and HT29) and a normal colorectal epithelial cell line (NCM460) (Figure 4(b)).

4. Discussion

In the present study, we used qRT-PCR, TMA-IHC, and bioinformatic analyses to detect LTBP2 mRNA and protein expression levels in CRC and reveal the relationship between LTBP2 expression and the clinical features of CRC patients. Our results showed that LTBP2 mRNA expression was significantly higher in CRC tissues than in matched adjacent tissues. LTBP2 protein expression was also higher in CRC tissues than in matched tumor-adjacent tissues and benign colorectal diseases. High LTBP2 protein expression was obviously correlated with higher TNM stage, higher T stage, higher N stage, and higher M stage. High LTBP2 protein expression was related to poor overall survival in CRC patients and could be seen as an independent prognostic factor for CRC.

LTBPs are key factors in regulating TGF-β activities. On one hand, LTBPs covalently link to SL-TGF-β and then participate in folding, assembling, secretion, localization, and activation of TGF-β [14, 15]. On the other hand, LTBPs promote TGF-β storage by binding fibrillin microfibrils in the extracellular matrix protein [16]. However, LTBP2 is unique, which means that LTBP2 does not covalently combine with latent TGF-β and indirectly mediates TGF-β activities by competing with LTBP1 for binding fibrillin microfibrils [10, 17]. TGF-β plays a bidirectional role in the occurrence and development of tumors. When the tumor occurs, TGF-β is a tumor suppressor through its growth inhibition activity;

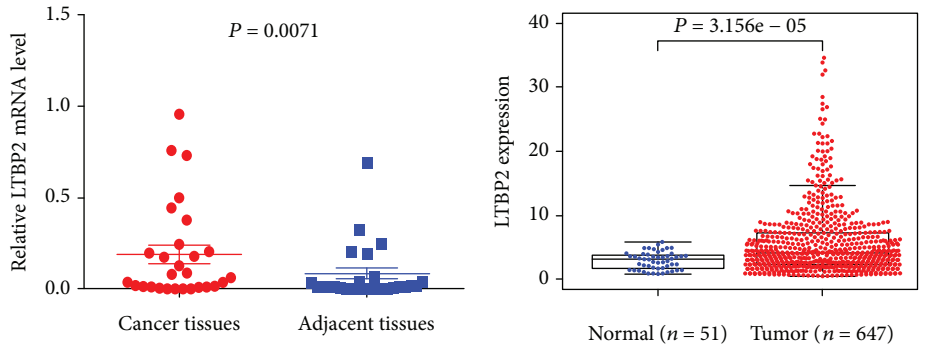

Figure 1: LTBP2 mRNA expression in CRC tissues and cell lines. (a) LTBP2 mRNA expression was significantly higher in CRC tissues than in matched adjacent tissues. LTBP2 mRNA was detected by qRT-PCR, and relative quantification analysis was normalized to GAPDH mRNA ($P = 0.0071$). (b) In TCGA database, LTBP2 mRNA expression was also higher in CRC tissues than in normal colorectal tissues ($P < 0.0001$).

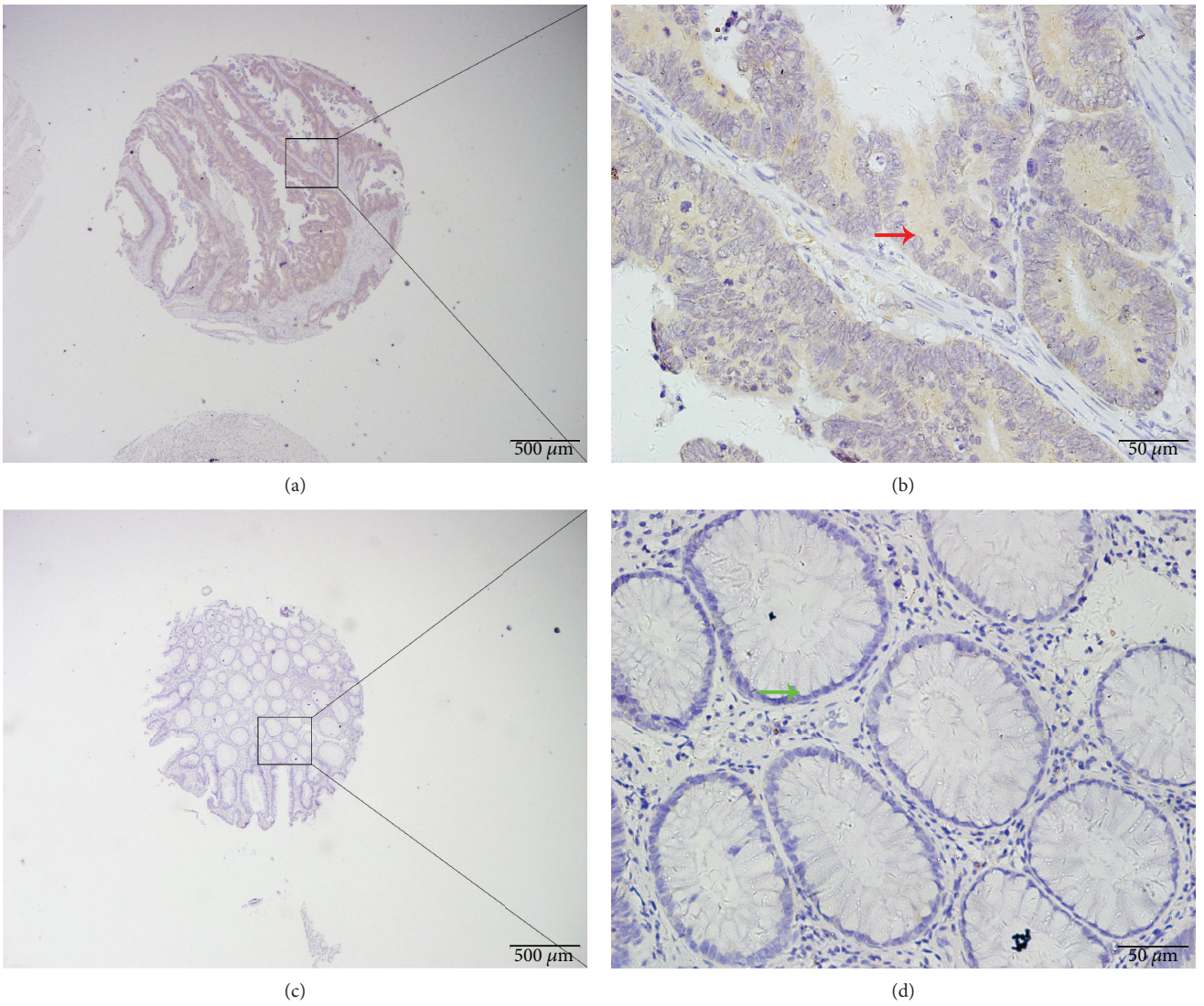

Figure 2: LTBP2 protein expression in CRC tissues. LTBP2 protein was determined by TMA-IHC. (a, b) Colorectal cancer with strong positive LTBP2 protein expression. (c, d) Adjacent normal tissue with negative LTBP2 protein expression. Red arrow represents positive LTBP2 protein expression in CRC tissue, and green arrow represents negative LTBP2 protein expression in adjacent normal tissue. Original magnification is ×40 (bar = 500 μm) in (a) and (c) and ×400 (bar = 50 μm) in (b) and (d).

Table 1: LTBP2 protein expression in CRC tissues and other tissues.

Feature	n	LTBP2		χ^2	P value
		Low or no expression	High expression		
Chronic colitis	23	22 (95.7%)	1 (4.3%)		
Low-grade intraepithelial neoplasia	44	41 (93.2%)	3 (6.8%)		
High-grade intraepithelial neoplasia	22	17 (77.3%)	5 (22.7%)		
Cancer	204	146 (71.6%)	58 (28.4%)		
Surgical margin[a]	190	177 (93.2%)	13 (6.8%)		
Total	483	403 (83.4%)	80 (16.6%)	39.896	<0.001*

[a]Epithelium without intraepithelial neoplasia from colorectal cancer. *$P < 0.05$.

Table 2: Correlation of LTBP2 protein expression with clinical characteristics of CRC patients.

Feature	n	LTBP2		χ^2	P value
		Low or no expression	High expression		
Total	204	146	58		
Gender				3.559	0.059
Male	127	85 (66.9%)	42 (33.1%)		
Female	77	61 (79.2%)	16 (20.8%)		
Age				0.001	0.976
≤60	63	45 (71.4%)	18 (28.6%)		
>60	141	101 (71.6%)	40 (28.4%)		
Location				1.343	0.511
Right colon	35	23 (65.7%)	12 (34.3%)		
Left colon	111	83 (74.8%)	28 (25.2%)		
Rectum	58	40 (69.0%)	18 (31.0%)		
Differentiation				0.095	0.954
Poor	4	3 (75.0%)	1 (25.0%)		
Well and middle	194	139 (71.6%)	55 (28.4%)		
Others[a]	6	4 (66.7%)	2 (33.3%)		
TNM stage				38.118	<0.001*
0 and I	47	44 (93.6%)	3 (6.4%)		
II	78	60 (76.9%)	18 (23.1%)		
III	65	40 (61.5%)	25 (38.5%)		
IV	14	2 (14.3%)	12 (85.7%)		
T stage				15.953	<0.001*
Tis, T1, and T2	54	50 (92.6%)	4 (7.4%)		
T3, T4	150	96 (64.0%)	54 (36.0%)		
N stage				20.443	<0.001*
N0	128	104 (81.2%)	24 (18.8%)		
N1a	40	26 (65.0%)	14 (35.0%)		
N1b	20	10 (50.0%)	10 (50.0%)		
N2a,b	16	6 (37.5%)	10 (62.5%)		
M stage				24.24	<0.001*
M0	190	144 (75.8%)	46 (24.2%)		
M1	14	2 (14.3%)	12 (85.7%)		
Preoperative CEA (ng/ml)				4.798	0.091
≤5	71	57 (80.3%)	14 (19.7%)		
>5	78	50 (64.1%)	28 (35.9%)		
Unknown	55	39 (70.9%)	16 (29.1%)		

[a]Mucinous adenocarcinoma, 6 cases. *$P < 0.05$.

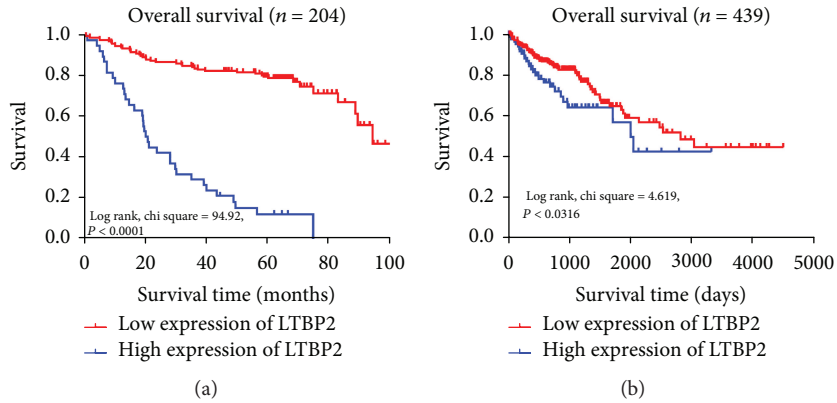

Figure 3: Survival curves of CRC patients by the Kaplan-Meier method and the log-rank test. (a) Patients with high LTBP2 expression (blue line) had significantly worse overall survival than those with low LTBP2 expression (red line). (b) In TCGA database, the high expression of LTBP2 (blue line) predicts poor overall survival for CRC patients.

Table 3: Univariate and multivariate analyses of prognostic factors for overall survival in CRC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Gender						
Male vs. female	1.591	0.966-2.619	0.068		NA	
Age						
≤60 vs. >60	1.008	0.0.617-1.648	0.973		NA	
Tumor location						
Right colon vs. left colon vs. rectum	1.038	0.773-1.393	0.806		NA	
Differentiation						
Poor vs. well and middle	0.533	0.177-1.607	0.264		NA	
TNM stage						
0 and I vs. II vs. III vs. IV	2.023	1.532-2.672	<0.001*	0.980	0.469-2.050	0.958
T stage						
Tis, T1, and T2 vs. T3 and T4b	3.398	1.692-6.825	0.001*	2.079	0.726-5.951	0.173
N stage						
N0 vs. N1a vs. N1b vs. N2a and N2b	1.493	1.207-1.847	<0.001*	1.044	0.734-1.486	0.809
M stage						
M0 vs. M1	5.983	3.244-11.036	<0.001*	1.986	0.665-5.934	0.219
Preoperative CEA (ng/ml)						
≤5 vs. >5	1.241	0.930-1.657	0.142		NA	
LTBP2 expression						
Low and none vs. high	23.619	13.036-42.794	<0.001*	21.056	11.274-39.326	<0.001*

Abbreviation: HR, hazard ratio; CI, confidence interval: NA, not considered in the multivariable model.

but during the process of tumor development, TGF-β can promote cell invasion, metastasis, angiogenesis, and immunosuppression [18–20]. Therefore, it is not difficult to guess that LTBP2 also has bilateral effects towards tumor development. For example, LTBP2 was more downregulated in NPC tumor tissues than in matched normal tissues and played a suppressive role in tumor development and progression [21]. LTBP2 was also epigenetically silenced in chronic lymphocytic leukemia and melanoma [22, 23]. On the other side, LTBP2 was upregulated in head and neck squamous cell carcinoma and was significantly related to lymph node metastasis and pTNM stage [24]. Patients with high LTBP2 expression had poorer survival in pancreatic carcinoma [15]. A mechanism study displayed that knockdown of LTBP2 inhibited the proliferation, migration, invasion, and epithelial-to-mesenchymal transition (EMT) of a phenotype of thyroid carcinoma cells [25]. Furthermore, LTBP2 is both tumor suppressing and tumor promoting in ESCC, which means that LTBP2 was more downregulated in tumor tissues than in matched normal tissues, but high LTBP2 predicts

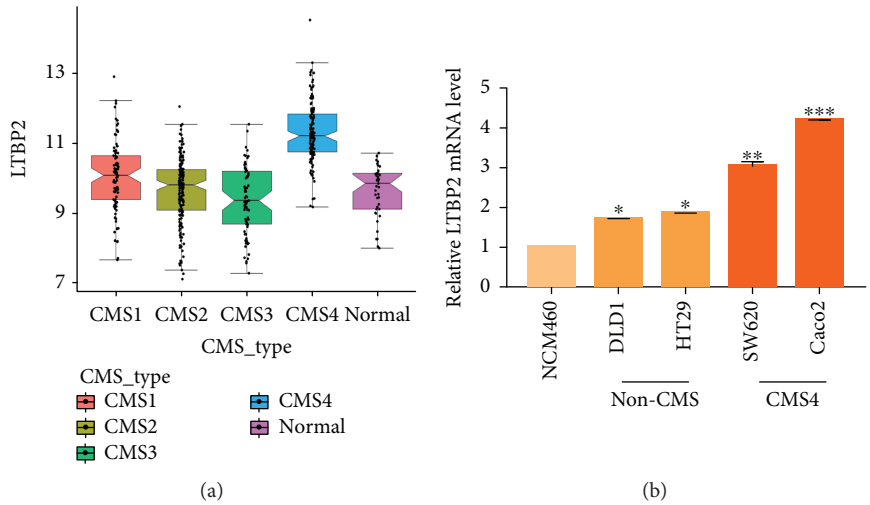

Figure 4: LTBP2 mRNA expression in the CMS4 subtype of CRC. (a) In TCGA database, LTBP2 mRNA expression was obviously higher in the CMS4 subtype than in other CRC subtypes ($P < 0.0001$). (b) LTBP2 mRNA expression was significantly higher in SW620 and Caco2 (CMS4) than in other CRC cell lines (non-CMS4) and in a normal colorectal epithelial cell (NCM460).

poor overall survival [26]. Our results suggest that LTBP2 may act as an oncogene in CRC and may predict poor prognosis for CRC patients.

CMS4 is considered to be an aggressive CRC subtype with a characteristic of overexpressing genes involved in epithelial-to-mesenchymal transition (EMT), TGF-β signaling, angiogenesis, and extracellular matrix remodeling [4, 5]. The roles of TGF-β in CRC tumorigenesis are multivariate and controversial in literature. Some studies assume that the main role of TGF-β is in the tumor stroma owing to the absence of expression in malignant epithelium, whereas other reports point out that active TGF-β signaling can be examined in epithelial tumor cells [27, 28]. No matter what the exact mechanism of TGF-β is, the increased activity of TGF-β is related to prognosis and the presence of metastatic lesions [29], which can be partially explained by the ability of TGF-β to induce EMT [30]. EMT is a process correlated with poor disease outcome, and the activation of EMT is a remarkable feature of CMS4 [31]. Thus, TGF-β signaling, a known inducer of EMT [32], can be active in CMS4. Considering the LTBP2 expression in CRC and the strong association between LTBP2 and TGF-β signaling [33], we conjectured that LTBP2 could be a novel biomarker for CMS4. Thus, we further analyzed 450 CRC samples in TCGA database and divided them into 4 groups (CMS1, CMS2, CMS3, and CMS4). Interestingly, LTBP2 was found to be specifically higher in the CMS4 subtype and was also overexpressed in CMS4 CRC cell lines. Unfortunately, we did not classify the specimens according to CMS in our study and analyze the LTBP2 expression in the CMS4 subtype due to the current limitation of the clinical application.

Our study also has several other limitations. Firstly, our clinical samples were obtained only from Chinese patients; therefore, the results may not be representative of other CRC populations. Larger international studies will be necessary to validate our findings. Secondly, the detailed mechanism of LTBP2 in CRC (especially in the CMS4 subtype) has not been revealed. It is necessary for us to further investigate the role of LTBP2 in the CMS4 subtype of CRC in the future.

In conclusion, our study demonstrates that high LTBP2 expression correlates with inferior survival in patients with CRC and plays a critical role in the progression of colorectal cancer. High LTBP2 expression predicts a poor outcome for CRC patients and could be considered as a novel biomarker and potential therapeutic target for the high-risk CMS4 subtype of colorectal cancer.

Data Availability

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

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Ying Huang, Guihua Wang, and Chunmei Zhao contributed equally to the manuscript.

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Review Article

Roles of Methylated DNA Biomarkers in Patients with Colorectal Cancer

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Colorectal cancer (CRC) is a leading cancer globally; therefore, early diagnosis and surveillance of this cancer are of paramount importance. Current methods of CRC diagnosis rely heavily on endoscopy or radiological imaging. Noninvasive tests including serum detection of the carcinoembryonic antigen (CEA) and faecal occult blood testing (FOBT) are associated with low sensitivity and specificity, especially at early stages. DNA methylation biomarkers have recently been found to have higher accuracy in CRC detection and enhanced prediction of prognosis and chemotherapy response. The most widely studied biomarker in CRC is methylated septin 9 (SEPT9), which is the only FDA-approved methylation-based biomarker for CRC. Apart from SEPT9, other methylated biomarkers including tachykinin-1 (TAC1), somatostatin (SST), and runt-related transcription factor 3 (RUNX3) have been shown to effectively detect CRC in a multitude of sample types. This review will discuss the performances of various methylated biomarkers used for CRC diagnosis and monitoring, when used alone or in combination.

1. Introduction

1.1. High Incidence and Mortality of Colorectal Cancer (CRC). Based on data from the GLOBOCAN study generated in 2012, the global incidence and mortality rates of CRC were shown to increase by 10-fold in a period of 10 years [1]. Specifically, CRC-related mortality is increasing rapidly in many low- and middle-income countries [1]. Furthermore, the incidence of CRC is predicted to continue to increase, especially in developing regions due to changing demographics and aging populations. When comparing the CRC incidence rates between 1988 and 2007 in eight regions globally, it is apparent that this increase is remarkable in both developing and developed countries except in America [2]. While screening for CRC among asymptomatic subjects is important, monitoring for CRC patients after treatment is also crucial. Hence, there is an urgent need to identify more robust early screening and detection biomarkers to facilitate the accurate early diagnosis and surveillance of this common malignancy.

1.2. Limitation of Recommended Tests. Although many methods exist for the diagnosis of colorectal cancer, the most accurate diagnostic method is generally considered to be colonoscopy with biopsy. Noninvasive diagnostic tests including blood and stool tests however seem to be more acceptable for screening of asymptomatic subjects as well as CRC patients for surveillance purposes. As yet, most of these noninvasive examinations have relatively low sensitivity and specificity, and false positive or negative results are not uncommon. Carcinoembryonic antigen (CEA) is the most widely used blood glycoprotein marker for CRC, particularly for monitoring of treatment response and surveillance. The American Society of Clinical Oncology has recommended testing of CEA every 3 months for at least 3 years following tumour resection in stages II and III CRC, while the European Group on Tumour Markers (EGTM) recommends testing for those who may receive liver resection or systemic treatment in a frequency of 2-3 months [3, 4]. However, a growing number of studies have casted doubt upon the role of using serum CEA in monitoring CRC recurrence due to

arbitrary thresholds used to depict the presence of disease in different studies [5–7]. Shinkins et al. reviewed 52 studies including 9,719 participants to determine the best CEA cut-off threshold, and all three selected thresholds were found to be unsatisfied. It was determined that threshold values of 2.5 μg/l or 5 μg/l produced many false positives (up to 20%), while values of 5 μg/l or 10 μg/l would result in nearly one-third of recurrences being left undiagnosed [6], deeming CEA as an unsatisfactory measure of CRC detection as alluded in other studies [8].

In regard to stool testing, faecal occult blood testing (FOBT) has been recommended for CRC screening in people 50 years or older by EGTM [4]. Unfortunately, FOBT was observed to have lower sensitivity in the proximal colon (71.2%; 95% CI: 61.3–79.4%) than distal colon (80.1%; 95% CI: 70.9–87.0%) [9]. Hence, it is necessary to discover more robust biomarkers for CRC screening and monitoring.

1.3. Methylated DNA Biomarkers. Discovery of epigenetic alterations in body fluids is an innovative alternative method of biomarker detection, with the advantages of stability, high frequency of positive detection, and noninvasive accessibility [10]. Among all studied epigenetic biomarkers, DNA methylation is the most frequently examined in various cancers, including CRC [11]. Methylated DNA biomarkers detected in CRC tissue, blood, and stool samples have been increasingly studied in recent years, but in many instances, the significance of their alterations in terms of functionality and biomarker value has not been properly characterized. Many studies have highlighted the potential of methylated DNA biomarkers for CRC detection and monitoring. Recently, methylated septin 9 (SEPT9) has been approved by the U.S. Food and Drug Administration (FDA) for screening of CRC [12]. Moreover, an increase in SEPT9 methylation levels in serum at 1-year follow-up after CRC resection may indicate potential recurrences. On the other hand, other methylated markers may also carry potential prognostic indications such as the methylated tachykinin-1 (TAC1) [13] and insulin-like growth factor binding protein 3 (IGFBP3) [14]. Detection of faecal methylated DNA has also been examined for CRC detection such as the eyes absent homolog 4 (EYA4) that was found to have a sensitivity of 100% (13/13) for CRC detection and 76.9% (27/35) for advanced adenoma, with a specificity of 94.7% (18/19) [15].

In this review, we summarized the performances of methylated markers for the diagnosis and surveillance of CRC (Table 1).

2. DNA Methylation Markers

2.1. Methylated Septin 9 (SEPT9). As the only methylated biomarker which has been approved for screening for CRC to date [12], serum SEPT9 has been studied extensively. In a recent systematic review, the second generation of SEPT9 was found to have a high sensitivity (71.1 to 95.6%) and specificity (81.5 to 99%) for CRC detection. When compared to faecal immunochemical test (FIT) in asymptomatic population, SEPT9 had an overall higher sensitivity (75.6% vs. 67.1%) and comparable specificity (90.4% vs. 92.0%) [29]. In our previous study, we found that the sensitivity of SEPT9 was significantly higher than CEA in detecting CRC (75.6% vs. 47.7%, $P < 0.001$) [16]. Monitoring SEPT9 biomarker use in CRC after surgical resection in a prospective cohort study of 150 CRC patients stages I-III, it was found that higher serum SEPT9 levels at 1 year and an increase in methylation from 6 months to 1 year and from preoperation to 1 year were indicative of a lower chance of disease-free survival [13]. Therefore, in addition to its approved diagnostic value, SEPT9 may have prognostic values in CRC.

2.2. Twist-Related Protein 1 (TWIST1). TWIST1 encodes a basic helix-loop-helix transcription factor, which promotes tumour cell invasion and metastasis in multiple human cancers [30]. In 2010, a Japanese study first reported altered TWIST1 methylation levels in different colorectal tissues, with the highest methylation levels in tumour and decreasing levels in colorectal adenoma and normal nontumour colorectal mucosa in CRC patients (median 55.7%, 25.6%, and 0.0%, respectively, $P < 0.001$). Methylated TWIST1 was suggested to be a potential biomarker in early CRC with a high accuracy for tissue detection of 89.6% [17]. Lin et al. examined 353 plasma samples from CRC patients through methylation-specific polymerase chain reaction (MSP) and found that 247 (70.0%) had TWIST1 hypermethylation. However, TWIST1 methylation was not found to have significant prognostic implication, with hazard ratios of 1.06 ($P = 0.799$) and 0.79 ($P = 0.463$), respectively, for univariate and multivariate analyses of disease-free survival [18]. Thus, although methylated TWIST1 is able to differentiate CRC from normal tissues, it may not be a reliable prognostic marker.

2.3. Runt-Related Transcription Factor 3 (RUNX3). RUNX3, a member of the RUNX family, has been shown to participate in various cancer pathways, including cell growth, apoptosis, and angiogenesis. RUNX associates with the Wnt oncogenic and TGF-β tumour suppressive pathways to promote CRC development [31]. The role of RUNX3 methylation has also been examined for diagnostic value in CRC in multiple studies [19–22, 32]. Huang et al. determined RUNX3 methylation levels in 30 colorectal cancer tissues and their paired adjacent normal tissues, showing that the RUNX3 methylation levels were significantly higher in tumour than in adjacent tissues (28% vs. 15%, $P < 0.01$) [19]. Shin et al. also observed that tissue RUNX3 hypermethylation had a sensitivity of 32.3% (20/62) and a specificity of 100.0% (0/10) for CRC detection. However, RUNX3 methylation levels were not found to be associated with stage ($P = 0.307$) and differentiation ($P = 0.179$) of tumours, but higher levels were linked with vascular ($P = 0.006$) and lymphatic ($P = 0.002$) invasions and worse prognosis ($P = 0.038$) [20]. In another study, hypermethylated RUNX3 was also detected in 41.5% (27/65) of CRC patients' serum samples [21]. Moreover, it was observed that a higher serum methylation level of RUNX3 was detected in patients with stages III and IV CRC than in healthy controls ($P = 0.0001$). In a 3-year follow-up study after resection of the primary tumour, the preoperative methylated levels of RUNX3 of 52 patients with recurrence were significantly

Table 1: DNA methylation biomarkers in detecting colorectal cancer.

Methylated gene	Sample type	Detecting method	No. of patients	Sensitivity*	No. of controls	Specificity*	*P* value for prognosis**	Reference
SEPT9	Blood	qPCR	90	75.6%	NA	NA	NA	[16]
	Serum	qMSP	150	NA	NA	NA	$P < 0.05$	[13]
TWIST1	Tissue	MSP	319	55.7%	215	100%	NA	[17]
	Plasma	MSP	353	70.0%	NA	NA	$P > 0.1$	[18]
RUNX3	Tissue	MSP	30	28%	30	85%	NA	[19]
	Tissue	MSP	62	32.3%	10	100%	$P = 0.038$	[20]
	Serum	MSP	65	41.5%	NA	NA	NA	[21]
	Serum	MSP	344	29%	56	100%	$P = 0.0003$	[22]
	Tissue	MSP	119	39%	NA	NA	NA	[22]
	Tissue	MSP	34	47%	17	88%	NA	[23]
TAC1	Serum	qMSP	150	NA	NA	NA	$P \leq 0.001$	[13]
	Serum	MSP	165	NA	NA	NA	$P = 0.612$	[24]
	Serum	MSP	193	NA	NA	NA	$P = 0.047$	[25]
IGFBP3	Tissue	qMSP	425	44.9%	21	NA	NA	[14]
	Tissue	MSP	147	NA	NA	NA	$P < 0.05$	[26]
	Tissue	MSP	115	NA	NA	NA	$P = 0.004$	[27]
EYA4	Tissue	MSP	46	93.5%	46	67.4%	NA	[15]
	Stool	MSP	13	100%	19	94.7%	NA	[15]
	Serum	qMSP	26	57.7%	26	≥90%	NA	[28]
SST	Serum	qMSP	150	NA	NA	NA	$P > 0.05$	[13]
	Tissue	MSP	34	88%	17	53%	NA	[23]
	Serum	qMSP	150	NA	NA	NA	$P < 0.05$	[13]
	Serum	MSP	165	NA	NA	NA	$P < 0.05$	[24]

*Sensitivity refers to the hypermethylation rates in colorectal cancer samples, while specificity refers to the opposite rates in normal samples. **P value for association of DNA hypermethylation with poorer prognosis, including cancer recurrence and reduced survival. SEPT9: methylated septin 9; TWIST1: twist-related protein 1; RUNX3: runt-related transcription factor 3; TAC1: tachykinin-1; IGFBP3: insulin-like growth factor binding protein 3; EYA4: eyes absent homolog 4; qPCR: quantitative polymerase chain reaction; qMSP: quantitative methylation-specific PCR; SST: somatostatin; NA: not available.

higher than that of 292 patients without recurrence ($P = 0.0003$) [22]. Hence, based on this investigation, RUNX3 not only has potential for CRC diagnosis but also may be useful in predicting CRC recurrence after operation.

2.4. Tachykinin-1 (TAC1). The tachykinins are a family of neuropeptides that share a common carboxyl terminus [33]. TAC1 is a member of this family and is the derivation of substance P and neurokinin A, which influence secretion, motility, and inflammatory reactions in the gastrointestinal tract [33]. The diagnostic potential of detecting and monitoring TAC1 methylation in CRC has been examined in a few studies [23–25]. Mori et al. found that methylated TAC1 was detected in 47% (16/34) of CRC tissue when compared to 12% (2/17) in normal colon mucosa ($P = 0.01$) [23]. Higher serum methylation levels of TAC1 6 months postoperation and an increasement of methylation levels during the first half-year interval were shown to be associated with cancer recurrence (both $P \leq 0.001$). When compared to serum CEA, the sensitivity of TAC1 for detecting recurrence was higher (58.1% vs. 32.6%, $P = 0.019$) at 6 months postresection and was able to detect CRC clinical recurrence 2.2 months prior to CEA [13]. However, two updated studies both demonstrated conflicting results and concluded that blood TAC1 hypermethylation was not a satisfactory biomarker for survival ($HR = 1.15$, $P = 0.612$ and $HR = 1.56$, $P = 0.047$) [24, 25].

2.5. Insulin-Like Growth Factor Binding Protein 3 (IGFBP3). IGFBP3 is one of the six homologous proteins which has high binding affinity with insulin-like growth factors I and II and can induce apoptosis and affect DNA synthesis [34]. The data on the association between IGFBP3 methylation and CRC however remains controversial. In a study carried out by Perez-Carbonell et al., IGFBP3 had higher diagnostic accuracy (83.0%) than five other markers (miR-137 78.3%, TWIST1 69.3%, SEPT9 65.8%, ALX4 61.6%, and GAS7 37.3%) for CRC, and low methylation levels of IGFBP3 indicated poor survival outcomes ($P = 0.01$). Contrastingly, in another study for stages II and III CRC patients who had received 5-fluorouracil- (5-FU-) based adjuvant chemotherapy, low IGFBP3 methylation levels were associated with longer overall survival ($P = 0.0007$) and disease-free survival ($P = 0.05$). In addition, chemotherapy did not enhance survival in patients with high IGFBP3 methylation levels [14]. Keeping with these findings, Fu et al. showed that the 5-year recurrence-free survival rate in stage II CRC with low methylation IGFBP3 was 3-fold higher than that of cases with high methylation (75.7% vs. 25.0%, respectively). Additionally, high IGFBP3 methylation levels in primary tumour were associated with recurrence ($P = 0.004$) [27]. Yi et al. also found that stage II CRC patients with tumour-methylated IGFBP3 had worse survival than those with unmethylated IGFBP3 ($P < 0.05$), and the former might benefit from adjuvant chemotherapy [26]. Hence, the clinical significance of IGFBP3 methylation levels remains controversial, and more studies are needed to characterize the importance of IGFBP3 as a prognostic marker.

2.6. Eyes Absent Homolog 4 (EYA4). Eyes absent (EYA) is a key regulator of ocular development in Drosophila, and EYA4 belongs to the family of its four homologues [35]. Methylated EYA4 is detectable in a variety of samples from CRC patients including serum, stool, and tumour tissue. Kim et al. detected tissue EYA4 methylation in CRC, paired normal colonic mucosae, and advanced adenoma, with positive rates of 93.5% (43/46), 32.6% (15/46), and 50.7% (36/71), respectively. They had also detected EYA4 methylation in stool samples and obtained a sensitivity of 83.3% (95% confidence interval (CI): 0.70-0.91) and a specificity of 94.7% (95% CI: 0.75-0.99) for diagnosing CRC and advanced adenoma [15]. Liu et al. detected serum methylated EYA4 in 26 Chinese patients with stage I CRC, with a sensitivity of 57.7% and a specificity of at least 90% [28]. However, serum EYA4 methylation was found to have no significant association with CRC recurrence or cancer-specific survival. EYA4 methylation in blood or tissue samples also had no association with radiological treatment for metastatic CRC [13, 36].

2.7. Somatostatin (SST). SST, a peptide synthesized in multiple tissues including the gastrointestinal tract, could act as a neurotransmitter or an inhibitory hormone [37]. Methylated SST was detected in 88% (30/34) of primary colorectal tumours, which was significantly higher than that in normal colon mucosae ($P < 0.001$) [23]. The level of SST methylation was also found to be higher in stage I CRC patients than in normal controls ($P = 0.037$). Serum methylated SST was significantly associated with cancer-specific survival among all other detected markers tested in the same study (TAC1, MAL, SEPT9, NELL1, CRABP1, EYA4, and CEA) at the preoperative time point, while its methylation status after operation had no value on prognosis [13, 28]. On the other hand, Liu et al. showed that the high serum SST methylation group had higher cancer recurrence after surgery than the low methylation group (38.7% vs. 18.7%, $P = 0.005$), and cancer-specific survival and disease-free survival were both longer in the latter as determined by univariate or multivariate Cox analysis (all $P < 0.05$) [24].

2.8. Combined Methylation Markers. Combining multiple methylated biomarkers may increase the diagnostic and prognostic accuracies for CRC. Perez-Carbonell et al. compared the accuracy of combined tissue methylated markers (TWIST1, IGFBP3, and miR-137) for the diagnosis of CRC. They found that the combined methylation markers increased the diagnostic accuracy to 92.0%, followed by miR-137+IGFBP3 (86.0%), IGFBP3 (83.0%), TWIST1 +IGFBP3 (82.7%), TWIST1+miR-137 (78.5%), miR-137 (78.3%), and TWIST1 (69.3%) [14]. Liu et al. demonstrated that the combination of serum methylation markers (TAC1 and EYA4) had a sensitivity level of 84.6% and a specificity of 80.8% for detecting CRC, while a combination of serum TAC1 and SEPT9 displayed an increased level of specificity of 92.3% with a sensitivity of 73.1% [28]. The combination of serum methylated TAC1, SEPT9, and NELL1 could also depict a higher cancer-specific death risk after CRC surgical resection ($P = 0.001$) than any single marker at 6 months but not after 1 year postsurgery [13].

CpG island methylator phenotype (CIMP) is a combination of methylation markers for diagnosis which has been studied extensively [38–43]. Early in 2009, Ogino et al. found that stages I-IV CRC patients who had CIMP-high tumour (defined as ≥6 of the 8 promoters positive: CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1) experienced significantly lower cancer-specific mortality [38]. However, some studies showed an inverse association between CIMP status and CRC prognosis. Vedeld et al. observed that CIMP-positive (≥3/5 promoters positive: CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1) CRC cases were significantly associated with a shorter recurrence time and worse overall survival after surgery [39]. Cha et al. also showed that the overall survival of metastatic CRC was longer in the lower-methylation group when eight markers were tested, with median survival of 9.77, 22.2, and 35.7 months for high CIMP (≥5), low CIMP (1-4), and CIMP-negative (0) groups, respectively ($P < 0.001$) [42]. The CIMP phenotype has also been monitored in response to chemotherapeutic agents with conflicting results. In some studies, improved prognosis was obtained for CRC patients with negative or low CIMP who received chemotherapy including 5-FU or/and oxaliplatin, while others found that positive or high CIMP was associated with better outcome after chemotherapy [40, 42]. Furthermore, some studies found no association between CIMP status with CRC chemotherapy [41, 43]. These conflicting results could be partly attributed to the inconsistent definition of CIMP in different studies.

2.9. Combination of DNA Methylation with CEA or FIT. Similar to the use of combined methylated markers, the combination of CEA or FIT with methylation markers could also improve the sensitivity of these tests for CRC. Suehiro et al. recently studied the diagnostic role of detecting TWIST1 methylation in faeces. The combined faecal TWIST1 methylation status tested together with FIT increased sensitivity to 82.4%, which was compared to the sensitivities of 47.1% and 41.2% when the test was used alone [44]. When compared to the use of a single marker, the combination of both CEA and CA19-9 with RUNX3 methylation provided higher sensitivity in the detection of CRC and did not reduce specificity [21].

These studies highlight the advantages of employing a combination of biomarkers to detect CRC. However, more studies are required to determine precisely which biomarkers should be selected and the optimal number of markers to be effective when considering the cost, complexity, and performance of these markers.

3. Current Issues Related to Detection of DNA Methylation

3.1. Tumour Characteristics. Methylated gene biomarkers are usually detected in tumours of higher staging, particularly in blood samples. Nishio et al. found that the average methylation ratio of RUNX3 in serum and tumour tissue increased with higher tumour stages ($P = 0.0466$ and $P = 0.0018$, respectively) [22]. By studying gene methylation distributions of 353 plasma samples from CRC patients of different tumour stages, Lin et al. found that AGBL4 (ATP/GTP binding protein-like 4) and FLI1 (friend leukaemia integration 1 transcription factor) methylation had the highest sensitivities in stage IV (77.8% and 81.0%, respectively) and lowest sensitivities in stage II (58.6% and 52.9%, respectively) [18]. Moreover, serum SST methylation was found to be a predictive value of cancer-specific survival in stage III patients as determined by multivariate Cox analysis ($HR = 2.52$, $P = 0.045$) but was not found to be significant in patients with stage II cancers ($P = 0.08$) [24].

Most studies showed that DNA methylation levels were usually associated with right-sided CRC. In a study conducted by Nishio et al., average tissue methylation levels of RUNX3 in the proximal colon were higher than that in the distal colon ($P = 0.0054$), but differential levels of sensitivity were not observed in serum methylation ($P = 0.2551$) [22]. Fu et al. observed higher IGFBP3 methylation in right-sided CRC as compared to left-sided CRC ($P < 0.001$) [27]. Additionally, Vedeld et al. found that CIMP-positive tumours were more frequently present in proximal CRC [39].

When comparing methylation levels of TAC1, SEPT9, NELL1, and SST in tumours and paired serum, methylation levels were consistently higher in tumours ($P < 0.05$) while there was no significant association of methylation statuses between the two sample types [13, 24].

3.2. Methods to Detect Methylation. Different methods to detect DNA methylation may alter the perceived methylation levels. Draht et al. compared four different methods in detecting CpG island methylation for 241 stage II CRC patients and found that nested-MSP had the highest sensitivity (33.1%) and was more effective compared to direct-MSP (10.7%), while pyrosequencing of 25% threshold obtained the best clinical specificity (90.2%), followed by methylation-sensitive high-resolution melting (87.7%) (Figure 1). However, there was no significant difference found in terms of prognostic implications when comparing different methods ($P > 0.05$) [45]. Recent studies have shown that droplet digital polymerase chain reaction (ddPCR) has advantages of increased precision, accuracy, and technical simplicity in comparison to conventional quantitative MSP, while high-resolution melting analysis was better than ddPCR in genotyping small deletion and insertion polymorphisms [46, 47]. However, results on their determination of methylated DNA from CRC patients are lacking.

3.3. Sample Type and Timing. In a recent study analyzing the SEPT9 methylation status of 9 CRC patients, plasma samples were collected at four separate times (06:00, 12:00, 18:00, and 24:00) in a day for testing. The results showed higher methylation values at 24:00 than any other time points (100% vs. 77.7%), and two stage I cases only had positive SEPT9 methylation at 24:00 [48]. Another earlier study explored the variation of DNA methylations of normal individuals after collecting 9 blood samples from each person at 3-hour intervals during 24 hours and discovered an increase of DNA methylation from 23:00 to 02:00 followed by a decline in

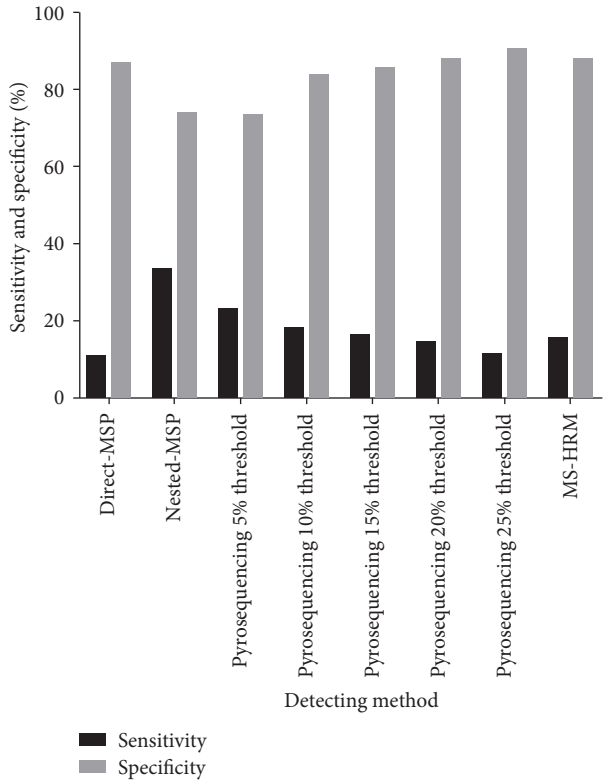

Figure 1: Clinical sensitivity and specificity for methylation detection through different methods. Sensitivity was calculated as $a/(a+b)$. Specificity was calculated as $d/(c+d)$. MSP: methylation-specific PCR; MS-HRM: methylation-sensitive high-resolution melting.

levels at 08:00 ($P = 0.021$). This was opposite to the trend observed in contemporaneous homocysteine levels, an amino acid which participates in the one-carbon metabolic pathway of DNA methylation for CRC, where there was no significant variation of its methylation status in the daytime [49, 50]. It may therefore be concluded that circadian variation of DNA methylation exists in CRC, which is probably related to cellular metabolic pathways. Larger-scale research of various methylated DNA biomarkers should be conducted to confirm these time-dependent observations.

4. Conclusion

Methylated genes have been shown to have potential in diagnosing, monitoring, and predicting chemotherapy response in CRC. The detection of methylated markers in serum/plasma or faecal samples represents a new, noninvasive method for cancer detection as well as a tool for monitoring during treatment. In addition, a combination of methylated markers has demonstrated an improved sensitivity and specificity of detection when compared to the currently used biomarkers CEA, FOBT, or FIT. Some internal and external factors including tumour stage, tumour location, and methylation detection technology can influence the perceived methylation levels; therefore, standardization of sample collection and methylation detection methods is required for clinical implementation in future studies.

Abbreviations

5-FU:	5-Fluorouracil
AGBL4:	ATP/GTP binding protein-like 4
ALX4:	Homeobox protein aristaless-like 4
CACNA1G:	Calcium voltage-gated channel subunit alpha1 G
CDKN2A (p16):	Cyclin-dependent kinase inhibitor 2A
CEA:	Carcinoembryonic antigen
CI:	Confidence interval
CIMP:	CpG island methylator phenotype
CRABP1:	Cellular retinoic acid-binding protein 1
CRC:	Colorectal cancer
ddPCR:	Droplet digital polymerase chain reaction
EGTM:	European Group on Tumour Markers
EYA4:	Eyes absent homolog 4
FDA:	U.S. Food and Drug Administration
FIT:	Faecal immunochemical test
FLI1:	Friend leukaemia integration 1 transcription factor
FOBT:	Faecal occult blood testing
GAS7:	Growth arrest specific 7
HR:	Hazard ratio
IGF2:	Insulin-like growth factor 2
IGFBP3:	Insulin-like growth factor binding protein 3
MAL:	Myelin and lymphocyte protein
MLH1:	MutL homolog 1
MSP:	Methylation-specific polymerase chain reaction
NELL1:	NEL-like protein 1
NEUROG1:	Neurogenin-1
RUNX3:	Runt-related transcription factor 3
SEPT9:	Methylated septin 9 DNA
SOCS1:	Suppressor of cytokine signaling 1
SST:	Somatostatin
TAC1:	Tachykinin-1
TWIST1:	Twist-related protein 1.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

ZM was involved in literature review, data collection, and drafting of the manuscript. MW helped revise the manuscript. YYC supervised and reviewed the manuscript. WKL was involved in the conception and critical review of the manuscript.

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