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Epigenetic inactivation of *SLIT2* in human hepatocellular carcinomas

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ABSTRACT

Recent findings have shown that *SLIT2* appears to function as a novel tumor suppressor gene. In addition, hypermethylation of its promoter region has been detected in various cancers, including breast and lung cancer, colorectal carcinoma, and gliomas. Here, we report for the first time that there is epigenetic silencing of *SLIT2* in human hepatocellular carcinoma (HCC). Downregulation of *SLIT2* was detected in 6 of 8 (75%) HCC cell lines by quantitative real-time RT-PCR (qRT-PCR), and the downregulation of *SLIT2* was generally dependent on the degree of methylation at the promoter region. Furthermore, expression of *SLIT2* was restored in relatively low-expressing cell lines after treatment with 5-aza-2-deoxycytidine (5-Aza-dC). Downregulation of *SLIT2* expression was also detected in 45 of 54 primary HCC samples (83.3%), and the decrease in expression was significantly correlated with CpG island hypermethylation. This decrease of *SLIT2* expression was also associated with lymph node metastasis in HCC. Moreover, overexpression of *SLIT2* in SMMC-7721 cells induced by recombinant adenovirus suppressed cell growth, migration, and invasion, These results suggest that epigenetic inactivation of *SLIT2* in HCC may be important in the development and progression of HCC. Thus, *SLIT2* may be useful as a therapeutic target in the treatment of HCC.

Human hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and annually accounts for up to 1 million deaths worldwide [1–4]. It is well known that heavy alcohol intake and infection with hepatitis B or C virus are important risk factors for HCC. In addition, numerous genetic abnormalities associated with the development of HCC have been described [5,6]. However, the detailed molecular mechanisms for the development and progression of HCC are not very clear.

Aberrant hypermethylation of CpG islands, which are CpG dinucleotide-rich areas located mainly in the promoter regions of many genes including tumor suppressor genes, DNA repair genes, and genes related to metastasis and invasion, is an alternative mechanism in the development of malignancies. Hypermethylation of gene promoters has been increasingly implicated as an early event in hepatocellular carcinogenesis [7,8]. The Slit family comprises large ECM-secreted and membrane-associated glycoproteins. Human Slits (*SLIT1*, *SLIT2*, and *SLIT3*) are candidate ligands for repulsive guidance receptors, the *ROBO* gene family. Slit-Robo interactions mediate the repulsive cues on axons and growth cones during neural development. As *SLIT2* is expressed in both neuronal and nonneuronal tissues, this suggested that it was potentially important outside a neural context. It has also been shown to inhibit leukocyte chemotaxis [9]. This inhibition can be controlled through the effect of *SLIT2* on actin cytoskeleton organization, mediated through Cdc42 [10].

Recent studies have shown that *SLIT2* is inactivated by promoter methylation in colorectal cancer, lung cancer, breast cancer, and gliomas [11–13]. However, hypermethylation of the *SLIT2* promoter has not been studied in HCC. In the present study, we used bisulfite sequencing and methylation-specific polymerase chain reaction (MSP) analysis to analyze the *SLIT2* methylation pattern in HCCs. The mRNA level of *SLIT2* in HCC cell lines and in HCCs was assessed by quantitative real-time RT-PCR (qRT-PCR). Subsequently, we investigated whether treatment of HCC cell lines hypermethylated at the *SLIT2* promoter with 5-aza-2-deoxycytidine (5-Aza-dC, a demethylating agent) could restore or increase expression of *SLIT2*. We found that the *SLIT2* promoter is frequently hypermethylated in HCC and that this epigenetic inactivation is the primary cause for *SLIT2* loss of function in HCC.

Materials and methods

Cell lines and samples. Eight HCC cell lines (SMMC-7721, HuH-7, MHCC97-L, HCCLM3, QGY-7701, BEL-7402, Hep3B, HepG2) and an

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immortalized human hepatocyte line (HepLL) were used. The cell lines were purchased from ATCC (American Type Culture Collection) or the Cell Bank of the Chinese Academy of Science. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Culture plates were maintained in a humidified incubator at 37 °C with 5% CO_2.

Fifty-four paired samples of HCC including tumor tissues and corresponding adjacent live non-cancerous tissues were collected from surgical specimens at The Qidong Liver Cancer Institute, The Oriental Liver and Gall Hospital, and The Cancer Hospital of Guangxi Medical University. Normal liver tissues ($n = 3$) were obtained from liver donors at The Liver Transplantation Unit in The First Affiliated Hospital, College of Medicine, Zhejiang University. Informed consent was obtained from every patient. Tissues were collected during surgery, immediately frozen in liquid nitrogen, and stored at −70 °C for DNA/RNA extraction. HCC diagnosis was confirmed by histology.

Bisulfite sequencing and MSP. Genomic DNA was extracted from cell lines and frozen tissues by standard protocol. Bisulfite treatment of genomic DNA was performed as described previously [14]. The *SLIT2* putative promoter region was predicted by Promoter Inspector software (http://www.dbtss.hgc.jp/) to be from −556 to −137 relative to the translation start site. The region was amplified by first-round PCR using the primers SLIT2L and SLIT2R1. Then, one-fiftieth of the PCR products (by volume) were used in nested PCRs using the primers SLIT2L and SLIT2R2. The sequences of primers are shown in Table 1. PCR were 95 °C for 10 min, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 52–56 °C, and 2 min extension at 72 °C. The PCR products were purified and cloned into pGEM-T Easy Vector (Promega). DNA sequencing was performed for at least five individual clones using a 377 automatic sequencer (Applied Biosystems).

The primer pairs for MSP were designed according to the DNA sequencing results. The sense and antisense primers for methylation-specific DNA (primers SLIT2-MF and SLIT2-MR, which amplified a 198 bp fragment) and for unmethylation-specific DNA (primers SLIT2-UF and SLIT2-UR, which amplified a 120 bp fragment) are shown in Table 1. PCR was conducted in a 12.5 μL reaction volume using kod-plus polymerase (ToYoBa). PCR conditions were as follows: 1 cycle at 95 °C for 10 min followed by 30 cycles of 94 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR products were separated on 2% agarose gels and visualized using UV illumination after ethidium bromide staining.

RNA isolation and qRT-PCR. Total RNA was isolated from each sample using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Five micrograms of total RNA from each sample was subjected to cDNA synthesis using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The expression level of *SLIT2* was detected by qRT-PCR, using the cDNA as the template, on an ABI 7300 Real-Time PCR System (Applied Biosystems). PCR reactions were performed in triplicate. Data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method [15]. The primers (rtSLIT2L and rtSLIT2R) used for qRT-PCR. are shown in Table 1. The PCR amplification cycles consisted of denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 90 s, annealing at 61 °C for 30 s, extension at 72 °C for 30 s, and a final elongation at 72 °C for 10 min. To minimize errors arising from variations in the amount of starting RNA in the samples, β-actin mRNA was used as an internal reference (primers β-actinL and β-actinR).

5-Aza-dC treatment. The demethylating agent 5-Aza-dC (Sigma) was freshly prepared in ddH_2O and filtered. Cells (5×10^5) were plated in a 60 mm dish in DMEM supplemented with 10% FBS. Twenty-four hours later, cells were treated with 10 μM 5-Aza-dC. RNA was extracted 10 days after treatment, and *SLIT2* expression was detected by qRT-PCR. β-Actin was amplified in the same PCR reactions as an internal control.

Preparation of recombinant adenovirus. The full-length cDNA construct of *SLIT2* was a gift from Dr. Yi Rao (Department of Neurology, Northwestern University, Feinberg Medical School, Chicago, IL, USA). The cDNA fragment was subcloned into the shuttle plasmid, pDC315 (Microbix Systems), which was cut using BamHI and SalI to produce the pDC315/*SLIT2* plasmid. Recombinant *SLIT2* adenoviruses (Ad-SLIT2) were generated using the Admax Cre/*lox*P system (Microbix Systems) according to the manufacturer's instructions. The control virus Ad-GFP was constructed in a similar manner. The virus titer was determined using an Adeno-X rapid titer kit (Clontech) according to the manufacturer's instructions.

Cell proliferation assay. SMMC-7721 cells were grown in 60 mm plates overnight and then infected with Ad-GFP or Ad-SLIT2 as described previously [16]. Briefly, SMMC-7721 cells at approximately 80% confluency were washed with serum-free DMEM and infected with Ad-GFP or Ad-SLIT2 at a titer of 2×10^8 particles/mL for 90 min. The medium was replaced by fresh DMEM containing 2% serum and incubated for 48 h. The SMMC-7721 cells were then cultured in 96-well flat-bottomed plates at 3×10^4 cells per well. The cell growth curve was assessed using the 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) assay (Sigma) according to the manufacturer's instructions. Each assay was performed at the indicated time points. The results were expressed as the absorbance at 570 nm.

Wound-healing assay. The SMMC-7721 cells infected with Ad-GFP or Ad-SLIT2 were plated at a density of 6×10^5 cells per well in six-well plates and allowed to reach suitable confluency. The cell monolayer was then gently wounded by scratching with a sterile 200 μL pipette tip. To remove cell debris, the wells were washed twice with PBS, and then incubated in DMEM with 2% FBS for 48 h. Then, the cells were washed in PBS, fixed in Carnoy's solution, and stained with crystal violet. The migrating cells were counted under the microscope. The experiment was performed in triplicate.

Invasion assay. The cell invasion ability was determined with the BioCoat Matrigel invasion chamber (BD Biosciences), in which the chamber membrane filter (8 μm pore size) was coated with BD Matrigel Basement Membrane Matrix (BD Biosciences). A total of 5×10^4 SMMC-7721 cells infected with either Ad-GFP or Ad-SLIT2 were seeded into the chamber in 24-well plates and incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. After removing the cells on the upper membrane, invading cells on the lower surface of the membrane were washed in PBS, fixed in Carnoy's solution, and stained with crystal violet. The invading cells were counted under the microscope in 10 randomly selected fields for each membrane filter (×100). The experiment was performed in triplicate.

Table 1
Primer sequences.

rtSLIT2L: 5′-GGTGTCCTCTGTGATGAAGAG-3′ rtSLIT2R: 5′-GTGTTTAGGAGACACACCTCG-3′
β-actinL: 5′-TTGTTACAGGAAGTCCCTTGCC-3′
β-actinR: 5′-ATGCTATCACCTCCCCTGTGTG-3′
SLIT2L: 5′-GGGAGGTGGGATTGTTTAGATATTT-3′ SLIT2R1: 5′CAAAAACTCCTTAAACAACTTTAAATCCTAAAA-3′ SLIT2R2: 5′-ACTAAAACTTCCAACAACTACTAAAATACAAAAA-3′
SLIT2-MF: 5′-GGGAGGCGGATTGTTTAG-3′ SLIT2-MR: 5′-CATAACGCGCGAAAATACAC-3′
SLIT2-UF: 5′-GTGGGAGGTGGATGTTTA-3′ SLIT2-UR: 5′-ACCTATCCCTCACCCTCAAC-3′

Statistical analysis. Comparisons were made by Student's *t*-test or the χ^2 test, where appropriate. *p* values of <0.05 or <0.01 were considered statistically significant.

Results

DNA methylation status of SLIT2 in HCC

Fig. 1 shows the results of bisulfite DNA sequencing and MSP analysis for *SLIT2* methylation status in HCC cell lines and clinical samples. Methylation of the *SLIT2* promoter was observed by MSP analysis in 6 of 8 HCC cell lines (75%) (Fig. 1C), 45 of 54 tumor tissues (83.3%), and 29 of 54 non-cancerous liver tissue (53.7%) from 54 HCC patients (Fig. 1D). However, *SLIT2* methylation was not detected in normal liver tissue from healthy controls or in an immortalized human hepatocyte line (HepLL) (Fig. 1C). For patients with HCC, hypermethylation of *SLIT2* CpG islands was more frequent in tumor tissues than in corresponding non-cancerous liver tissues ($p < 0.05$).

SLIT2 expression in HCC

We examined the *SLIT2* expression status in nine cell lines (Hep3B, HepG2, HUH-7, MHCC97-L, HCCLM3, SMMC-7721, QGY-7701, BEL-7402, and HepLL) and three normal liver tissues by qRT-PCR. The relative mRNA expression in each cell line is shown in Fig. 2A. Expression ratios of methylated cell lines were much less than those of unmethylated cell lines. These results indicated that the expression of *SLIT2* tended to be reduced depending on the degree of methylation at the promoter region. In HepLL cells, the mean expression ratio was 75.1%, indicating that *SLIT2* was normally expressed in this immortalized human hepatocyte line.

To further examine *SLIT2* expression in HCC clinical samples, we analyzed a cohort of 54 paired samples obtained from patients with HCC, including tumor tissues and adjacent non-cancerous liver tissues. In 33 patients with HCC (33/54), the mRNA level of *SLIT2* in tumor tissues was significantly reduced compared to corresponding adjacent non-cancerous liver tissue ($p < 0.01$). In 12

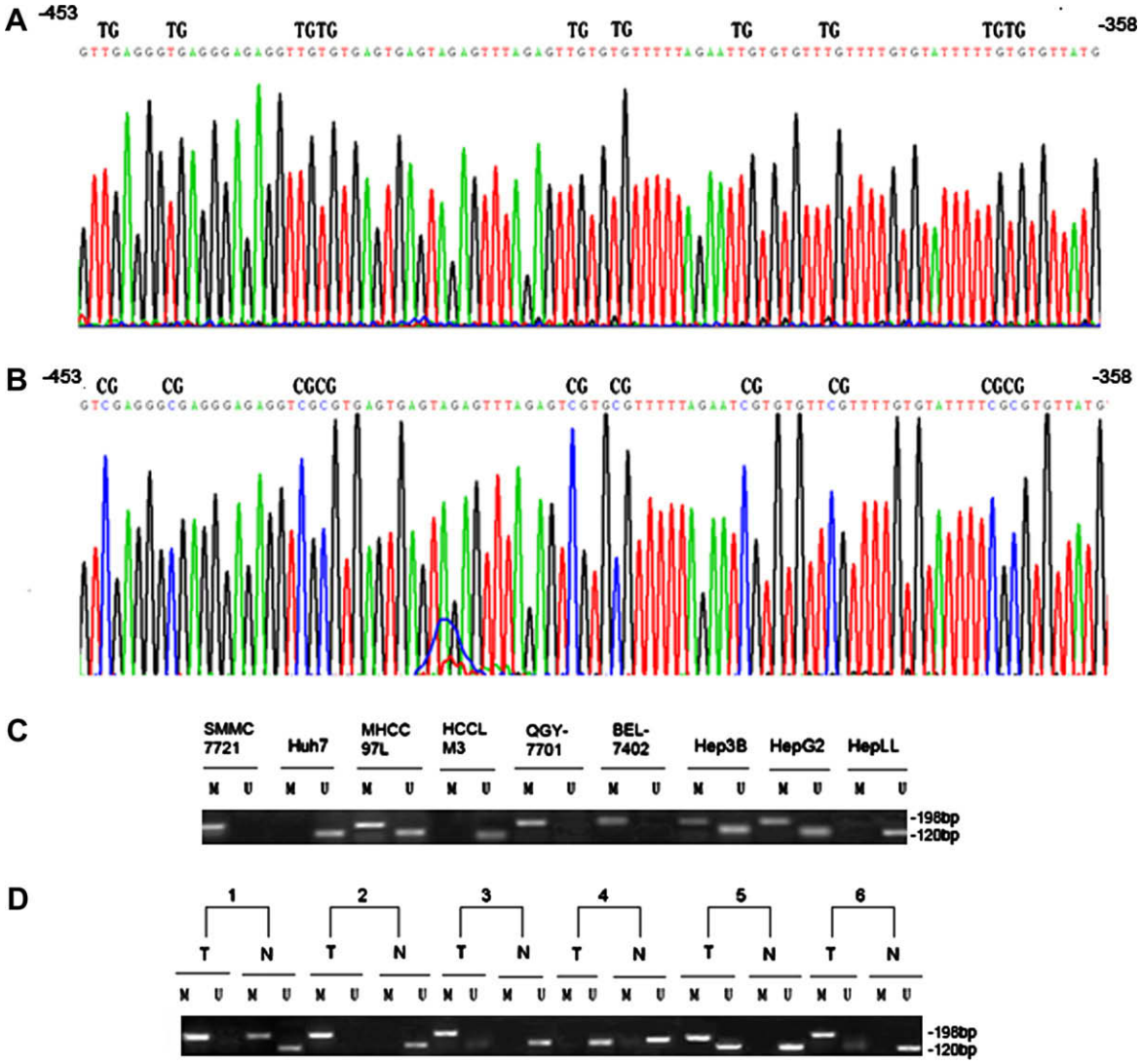

Fig. 1. Methylation analysis of *SLIT2* in HCC and cell lines. T: tumor tissues; N: adjacent non-cancerous liver tissues; M: methylation-specific primers; U: unmethylation-specific primers. (A) Representative results of sequencing of bisulfite-modified DNA from tumor tissues with an unmethylated *SLIT2* CpG island. The locations of unmethylated CpGs are indicated by TG. (B) Representative results of sequencing of bisulfite-modified DNA from tumor tissues with a methylated *SLIT2* CpG island. The locations of methylated CpGs are indicated by CG. (C) MSP in cell lines. (D) Representative examples of MSP in tumor tissues and their corresponding adjacent non-cancerous liver tissues.

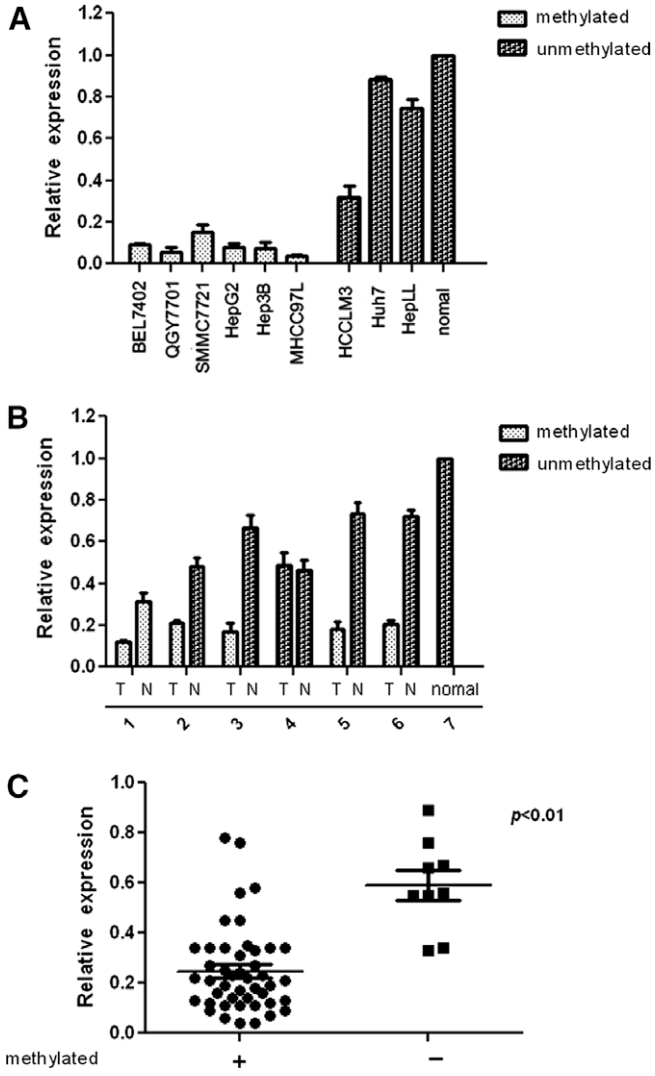

Fig. 2. Expression levels of *SLIT2* mRNA in cell lines and HCC. Data shown are mean values ± SD, $n = 3$. T: tumor tissues; N: adjacent non-cancerous liver tissues; normal: normal liver tissue. (A) The relative ratio of *SLIT2* mRNA in cell lines. In two unmethylated cell lines, the mean *SLIT2* expression ratios were 33.6% (HCCLM3) and 88.0% (HuH-7). The expression ratios of methylated cell lines were much lower than those of unmethylated cell lines (11% in BEL-7402, 5.2% in QGY-7701, 15.2% in SMMC-7721, 7.3% in HepG2, 6.7% in Hep3B, and 4.1% in MHCC97-L). In HepLL cells, the mean expression ratio was 75.1%. (B) The relative ratio of *SLIT2* mRNA in tumor tissues and corresponding adjacent non-cancerous liver tissues in HCC. The number below T and N is the code of patients with HCC. (C) The relative ratio of *SLIT2* expression in methylated and unmethylated tumor tissues in patients with HCC.

patients with HCC (12/54), the mRNA level was not significantly different between tumor and adjacent non-cancerous liver tissues, and only 9 patients (9/54) had higher mRNA levels of *SLIT2* in their tumor tissues compared with adjacent non-cancerous liver tissue. Representative examples are shown in Fig. 2B. The expression of *SLIT2* tended to be reduced depending on the degree of methylation at the promoter region in HCC ($p < 0.01$) (Fig. 2C).

5-Aza-dc treatment for HCC cell lines

To explore the role of DNA methylation in *SLIT2* silencing, we treated two HCC cell lines, SMMC9721 and MHCC97-L, with 5-Aza-dc. In the two methylated cell lines, *SLIT2* expression was significantly upregulated by 5-Aza-dc treatment (>3.5-fold) (Fig. 3).

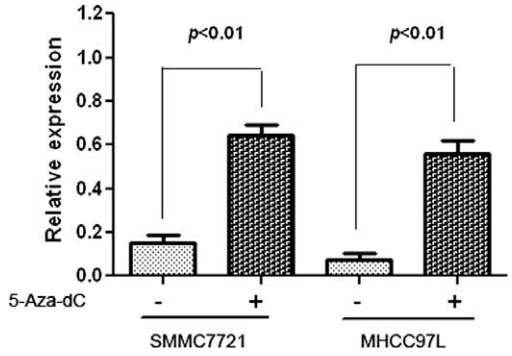

Fig. 3. Effect of 5-aza-deoxycytidine (5-Aza-dC) on *SLIT2* expression in methylated cell lines (SMMC-7721 and MHCC97-L). *SLIT2* expression was dramatically upregulated by 5-Aza-dC in the two cell lines (3.5-fold for SMMC-7721, $p = 0.0015$; 5.5-fold for MHCC97-L, $p = 0.0024$).

The effects of SLIT2 overexpression on cell growth, migration, and invasion in HCC cells

Since *SLIT2* overexpression suppresses cell migration in vascular smooth muscle cells [17], we examined whether *SLIT2* has the same effect on cell migration in HCC cells. As shown in Fig. 4A, the SMMC-7721 cells infected with Ad-SLIT2 grew more slowly than the control cells ($p < 0.05$). We also found that SMMC-7721 cells infected with Ad-SLIT2 migrated less than the control cells ($p < 0.01$) (Fig. 4B). Furthermore, overexpression of *SLIT2* could significantly suppress cell invasion in SMMC-7721 cells ($p < 0.01$) (Fig. 4C). These results suggest that *SLIT2* may be associated with cell growth, cell migration, and invasion in HCC cell lines.

Correlation of gene methylation with clinicopathologic variables in HCC

We next assessed the relationship between the methylation status of *SLIT2* and eight clinicopathologic factors in HCC; namely gender, age, hepatitis B infection status, tumor size, vascular invasion, degree of underlying cirrhosis, tumor differentiation, and lymph node metastasis (Table 2). We observed that downregulation of *SLIT2* by promoter methylation correlated with lymph node metastasis in HCC. No significant correlations were observed between *SLIT2* methylation and the other clinicopathologic factors.

Discussion

Promoter hypermethylation has recently been shown to be a common inactivation mechanism for tumor suppressor genes in human cancers [18–20]. Hypermethylation of the promoter region in *SLIT2* has been detected in various cancers [11–13]. In this study, we report that *SLIT2* is a target for methylation and inactivation in HCC. Methylation of *SLIT2* was associated with loss of expression in HCC cell lines, where this loss was reversed by treatment with a demethylating agent. Further, the promoter region of *SLIT2* was methylated in 83.3% of primary HCCs. Promoter methylation of *SLIT2* was well correlated with a reduction of expression in HCC clinical samples. These results indicate that DNA methylation of the promoter is critical for *SLIT2* transcription and that methylation plays an important role in *SLIT2* inactivation. Promoter methylation (53.7%) in *SLIT2* was detected in histologically normal corresponding non-cancerous liver tissues. This result may indicate that promoter methylation of *SLIT2* occurs very early in cancer progression. However, it is possible that we were simply detecting cancer cells with methylated *SLIT2* that had invaded into corresponding adjacent non-cancerous liver tissues.

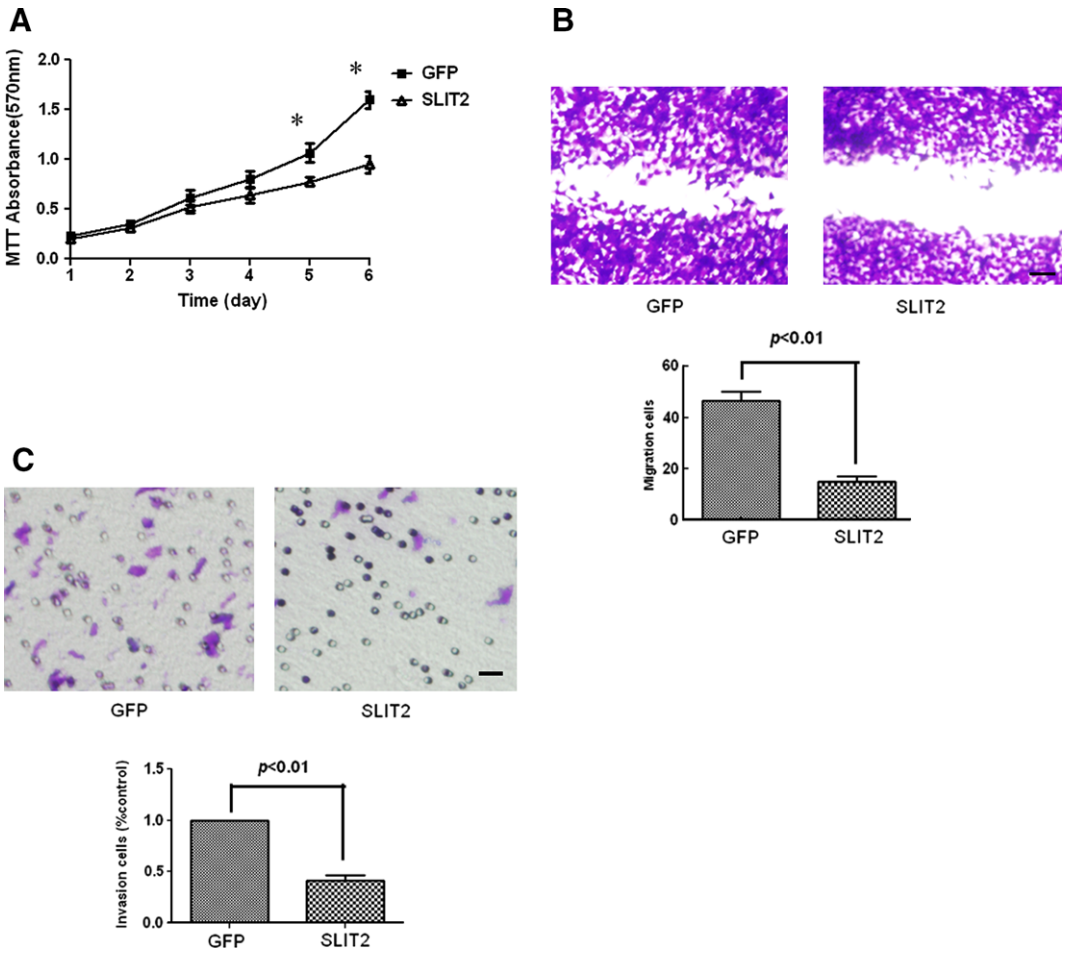

Fig. 4. Effect of *SLIT2* overexpression on cell growth, migration, and invasion. (A) Cell proliferation assay. SMMC-7721 cells were infected with Ad-GFP (control) or Ad-SLIT2. The SMMC-7721 cells infected with Ad-SLIT2 grew more slowly than the control. Each point represents the mean ± SD of triplicate determinations. Statistically significant differences ($^{*}p < 0.05$) were obtained ($n = 6$ wells). (B) Cell migration assay. SMMC-7721 cells infected with either Ad-GFP (control) or Ad-SLIT2 were photographed after 48 h. There were significantly less migrating cells among those infected with Ad-SLIT2 compared to control ($p < 0.0001$). Bar indicates 150 nm. (C) Cell invasion assay. SMMC-7721 cells infected by either Ad-GFP (control) or Ad-SLIT2 were seeded in Matrigel-coated transwells and allowed to migrate for 48 h. There were significantly less migrating cells in those infected with Ad-SLIT2 compared to control ($p = 0.0073$). Bar indicates 40 nm.

Table 2
Clinicopathological characteristics and methylation status of the *SLIT2* promoter in HCC patients.

Clinicopathological characteristics	Variable	No. of cases	*SLIT2* methylation		*p*
			+	–	
Gender	Male	45	38	7	>0.05
	Female	9	7	2	
Age (y)	<60	33	27	6	>0.05
	⩾60	21	18	3	
Hepatitis virus infection	–	16	14	2	>0.05
	HBV	27	23	4	
	HCV	11	8	3	
Tumor size	<3 cm	20	17	3	>0.05
	⩾3 cm	34	28	6	
Tumor differentiation	I	21	17	4	>0.05
	II	12	9	3	
	III	21	19	2	
Vascular invasion	–	19	13	6	>0.05
	+	35	32	3	
Liver cirrhosis	–	41	35	6	>0.05
	+	13	10	3	
Lymph node metastasis	–	23	16	7	<0.05
	+	31	29	2	

We found that restoration of *SLIT2* expression has a variety of effects on HCC cell lines including suppressing growth of HCC cells and reducing cell migration and invasion *in vitro*. Combined with the correlation between downregulation of *SLIT2* by promoter methylation and lymph node metastasis in HCC (Table 2), our findings indicate that *SLIT2* is not only a tumor suppressor but also a metastasis suppressor in HCC. However, the molecular mechanisms of this remain unclear. Recently, Marlow et al. observed that loss of *Slits* in human breast carcinoma cells results in upregulation of the *Sdf1* and *Cxcr4* signaling axis [21]. The interaction of *Sdf1* and *Cxcr4* promote cell growth, enhance tumor migration and invasion, and contribute to tumor metastasis within the tumor microenvironment [22–23]. Further study is necessary to elucidate the mechanism of how *SLIT2* inhibits cell growth and migration in HCC.

Metastasis is the main cause of death in cancer patients. It has been described that *SLIT2* plays an important role in tumor angiogenesis by blocking VEGF induced migration of HUVECs [24] and *SLIT2* deletion in chromosome 4 is significantly associated with poor survival of the patients with cervical cancer [25]. In this report our data indicate that downregulation of *SLIT2* by promoter methylation is correlated with lymph node metastasis in HCC and imply that the HCC patients with promoter methylation of

SLIT2 may have poor prognosis. These findings suggest that *SLIT2* may be a potential prognostic factor for cancer patients.

In summary, our results suggest that *SLIT2* may be a candidate tumor and metastasis suppressor and that it is frequently inactivated by promoter methylation in HCC. As it is functionally involved in cancer progression and metastasis, *SLIT2* may be considered as a possible therapeutic target in the treatment of HCC.

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References

[1] A.S. Befeler, A.M. Di Bisceglie, Hepatocellular carcinoma: diagnosis and treatment, Gastroenterology 122 (2002) 1609–1619.
[2] H.B. El-Serag, Hepatocellular carcinoma: recent trends in the United States, Gastroenterology 127 (2004) S27–S34.
[3] H.B. El-Serag, AC. Mason, Rising incidence of hepatocellular carcinoma in the United States, N. Engl. J. Med. 340 (1999) 745–750.
[4] F.X. Bosch, J. Ribes, R. Cleries, M. Diaz, Epidemiology of hepatocellular carcinoma, Clin. Liver Dis. 9 (2005) 191–211.
[5] Y.S. Kahng, Y.S. Lee, B.K. Kim, W.S. Park, J.Y. Lee, C.S. Kang, Loss of heterozygosity of chromosome 8p and 11p in the dysplastic nodule and hepatocellular carcinoma, J. Gastroenterol. Hepatol. 18 (2003) 430–436.
[6] M.A. Buendia, Genetics of hepatocellular carcinoma, Semin. Cancer Biol. 10 (2000) 185–200.
[7] J. Yu, H.Y. Zhang, Z.Z. Ma, W. Lu, Y.F. Wang, J.D. Zhu, Methylation profiling of twenty four genes and the concordant methylation behaviours of nineteen genes that may contribute to hepatocellular carcinogenesis, Cell Res. 13 (2003) 319–333.
[8] B. Yang, M. Guo, J.G. Herman, D.P. Clark, Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma, Am. J. Pathol. 163 (2003) 1101–1107.
[9] J.Y. Wu, L. Feng, H.T. Park, N. Havlioglu, L. Wen, H. Tang, K.B. Bacon, Z. Jiang, X. Zhang, Y. Rao, The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors, Nature (London) 410 (2001) 948–952.
[10] K. Wong, X.R. Ren, Y.Z. Huang, Y. Xie, G. Liu, H. Saito, H. Tang, L. Wen, S.M. Brady-Kalnay, L. Mei, J.Y. Wu, W.C. Xiong, Y. Rao, Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway, Cell 107 (2001) 209–221.
[11] A. Dallol, NF. Da Silva, P. Viacava, J.D. Minna, I. Bieche, E.R. Maher, F. Latif, SLIT2, a human homologue of the *Drosophila* Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers, Cancer Res. 62 (2002) 5874–5880.
[12] A. Dallol, D. Krex, L. Hesson, C. Eng, E.R. Maher, F. Latif, Frequent epigenetic inactivation of the SLIT2 gene in gliomas, Oncogene 22 (2003) 4611–4616.
[13] A. Dallol, D. Morton, E.R. Maher, F. Latif, SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells, Cancer Res. 63 (2003) 1054–1058.
[14] J.G. Herman, J.R. Graff, S. Myohanen, B.D. Nelkin, S.B. Baylin, Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands, Proc. Natl. Acad. Sci. USA 93 (1996) 9821–9826.
[15] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method, Methods 25 (2001) 402–408.
[16] M. Schuler, E. Bossy-Wetzel, J.C. Goldstein, P. Fitzgerald, D.R. Green, p53 induces apoptosis by caspase activation through mitochondrial cytochrome *c* release, J. Biol. Chem. 275 (2000) 7337–7342.
[17] D. Liu, J. Hou, X. Hu, Neuronal chemorepellent Slit2 inhibits vascular smooth muscle cell migration by suppressing small GTPase Rac1 activation, Circ. Res. 98 (2006) 480–489.
[18] S.B. Baylin, J.G. Herman, DNA hypermethylation in tumorigenesis: epigenetics joins genetics, Trends Genet. 16 (2000) 168–174.
[19] R. Dammann, C. Li, J.H. Yoon, P.L. Chin, S. Bates, G.P. Pfeifer, Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3, Nat. Genet. 25 (2000) 315–319.
[20] A. Agathanggelou, S. Honorio, D.P. Macartney, A. Martinez, A. Dallol, J. Rader, P. Fullwood, A. Chauhan, R. Walker, J.A. Shaw, S. Hosoe, M.I. Lerman, J.D. Minna, E.R. Maher, F. Latif, Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours, Oncogene 20 (2001) 1509–1518.
[21] R. Marlow, P. Strickland, J.S. Lee, X. Wu, M. PeBenito, M. Binnewies, E.K. Le, A. Moran, H. Macias, R.D. Cardiff, S. Sukumar, L. Hinck, SLITs suppress tumor growth in vivo by silencing Sdf1/Cxcr4 within breast epithelium, Cancer Res. 68 (2008) 7819–7827.
[22] J.A. Burger, T.J. Kipps, CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment, Blood 107 (2006) 1761–1767.
[23] Y. Alsayed, H. Ngo, J. Runnels, X. Leleu, U.K. Singha, C.M. Pitsillides, J.A. Spencer, T. Kimlinger, J.M. Ghobrial, X. Jia, G. Lu, M. Timm, A. Kumar, D. Cote, I. Veilleux, K.E. Hedin, G.D. Roodman, T.E. Witzig, A.L. Kung, T. Hideshima, K.C. Anderson, C.P. Lin, I.M. Ghobrial, Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma, Blood 109 (2007) 2708–2717.
[24] P. Seth, Y. Lin, J. Hanai, V. Shivalingappa, M.P. Duyao, V.P. Sukhatme, Magic roundabout, a tumor endothelial marker: expression and signaling, Biochem. Biophys. Res. Commun. 332 (2005) 533–541.
[25] R.K. Singh, D. Indra, S. Mitra, R.K. Mondal, P.S. Basu, A. Roy, S. Roychowdhury, C.K. Panda, Deletions in chromosome 4 differentially associated with the development of cervical cancer: evidence of slit2, Hum. Genet. 122 (2007) 71–81.