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Aberrant methylation status of SPG20 promoter in hepatocellular carcinoma: A potential tumor metastasis biomarker

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Abstract

Purpose: The aim of this study is to analyze the methylation levels of SPG20 promotor region and explore the association between the methylation levels and clinical features in hepatocellular carcinoma (HCC).

Materials and methods: We collected paired of HCC and adjacent non-cancerous tissues (ANT) from 160 HCC patients and analyze the methylation levels through MassARRAY Analyzer 4. The statistical calculations were performed using SPSS version 22.0. Real-time-quantification PCR was performed to assess expression levels of SPG20 in HCC cell lines. Wound healing assay and transwell assay was used to measure cell migration capacity.

Result: We found that mean methylation level of SPG20 in tumor tissues was significantly higher than that in ANT (7.3% vs. 16.2%, $P<0.0013$). There was a significantly negative correlation between expression level and methylation level of SPG20 ($P<0.01$). In addition, the methylation levels in HCC were correlated with age and HBV infection. Meanwhile, micro-satellite tumors ($P=0.016$) and tumor number ($P=0.018$) was found significantly associated with increased methylation levels of several CpG sites and the mean levels of SPG20 promotor in ANT. In addtion, the capacity of cell migration was significantly enhanced in SPG20 knock-down HCC cells.

Conclusion: The hypermethylation status of SPG20 gene promoter is significantly associated with intra-hepatic metastasis and contribute to HCC metastasis.

Keywords SPG20, Hepatocellular carcinoma, Methylation, Metastasis.

Introduction

Hepatocellular carcinoma (HCC) is the sixth highest malignant tumor in the world. Because of its complicated pathogenesis, high recurrence, metastasis rate and tumor heterogeneity, HCC has become the third most frequent cause of cancer-related death [1]. However, the 5-year survival of HCC patients can achieve higher than 50% when diagnosis at early stages [2].Therefore, a better understand of the pathogenesis for HCC is critical to improve the prognosis of patients with HCC through early diagnosis and treatment.

Recently, more and more evidences reveal that epigenetic dysregulation plays a driver role in the origin of tumorigenesis

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and can be used as prognostic and predictive biomarkers in oncology [3]. DNA methylation is the best studied epigenetic modification, and the hypermethylation of gene promoters may disrupt the function by silencing gene expression [4]. In HCC, the aberrant DNA-methylation in the promoters of some described tumor suppressors or potential oncogenes are confirmed and could be a prognostic biomarker [5,6].

The SPG20 gene which encodes spartin locates in chromosome band 13q13.3, and its mutation lead to the down-regulated expression of spartin which is a core factor resulting in the Troyer syndrome [7]. Spartin comprehensively associates with various cellular organelles. It has been verified to participate in transportation of epidermal growth factor receptor (EGFR) [8] and metabolism of lipid droplet [9]. It has also been reported that spartin plays a regulator of cytokinesis role in cell cycle [10]. The downregulation of spartin results in cytokinesis arrest and could induce the aneuploidy chromosomes in cells which might further lead to carcinogenesis. Liver is a specific organ with high proportion of four, eight and other polyploid cells. Although the polyploid cells protect liver from xenobiotic or nutritional injury, they also give rise to increased genomic instability and tumorigenesis [11]. When the liver suffers from injury, polyploidy may transfer to aneuploidy which is associated with tumorigenesis. Cytokinesis failure is considered to play a significant role in forming hepatocyte aneuploidy [12]. Previous study presented the hypermethylation status of SPG20 promoter down-regulated the expression of spartin cause the cytokinesis arrest in colorectal carcinomas, which suggests it might play a potential role in genomic instability and could be a sensitive biomarker for early stage colorectal tumors [13]. Therefore, we proposed the SPG20 gene might also play a specific role in HCC initiation.

Here, we firstly confirmed the methylation differences of the SPG20 promoter between cancerous and adjacent benign liver tissues from 160 HCC patients. Then the methylation levels of SPG20 in HCC were found age and HBV infection related. Moreover, we evaluated the association between the methylation alteration of SPG20 gene promoter and the clinical characteristics. We found the SPG20 methylation was correlated with multi-satellite tumors and tumor number and might contribute to HCC metastasis.

Materials and methods

Patient samples

During July 2008 to February 2014, we collected human primary HCC tissues and paired adjacent noncancerous tissues (ANT) from 111 patients treated at the Zhejiang Cancer Hospital and 49 patients treated at the Sir Run Run Shaw Hospital. The detailed information of the patients was summarized in Table 1. All tissues were diagnosed as HCC by pathology according to criteria of morphology and immunohistochemistry provided by Word Health Organization (WHO). All tissues were frozen in liquid nitrogen immediately and stored at −80°C for further extracting DNA and RNA. The study process was approved by the Ethics Committee of the Sir Run Run Shaw Hospital and the Ethics Committee of Zhejiang Cancer hospital. Informed consent was obtained from all patients.

Table 1 Patients characteristics.

Parameters	Categories	NO.
Sex	Female	29
	Male	136
Alcohol habit	No	105
	Yes	55
Hepatitis	None	22
	HBV	138
High blood pressure	No	120
	Yes	40
Type 2 diabetes	No	140
	Yes	20
Family history	No	143
	Yes	17
Age(mean ± SD)	54.0 ± 12.1	
AFP(mean ± SE)	2507.80±537.64	
Diameter of tumor (mean ± SE)	17.35±1.80	
Fibrous capsule	No	52
	Yes	108
Satellite tumor	No	145
	Yes	15
Tumornecrosis	No	131
	Yes	29
Microvascular invasion	No	129
	Yes	31
Liver cirrhosis	No	13
	Yes	128
TNM stage	No	114
	Yes	45

DNA extraction and bisulfite conversion

Genomic DNA was extracted from ≥25 mg primary HCC tissues and paired ANT tissues using the QIAamp DNAMini Kit refer to the standard protocol (QIAGEN, Hilden, Germany). All of the DNA samples were detected the concentrations and quality by Thermo NanoDrop2000. Then, we took 400–500 ng of DNA for bisulfite treatment using the EpiTect Fast DNA Bisulfite Kit (QIAGEN, Hilden, Germany).

Cell culture, RNA isolation and real-time-quantification PCR

Seven hepatoma cell lines SMCC-7721, LM3, Huh7, SK-Hep1, Hep-G2, PLC/PRF/5, JHH7 and Snu-423 and a non-tumour human hepatic LO2 cell line was used to analyze the expression levels of SPG20. SK-Hep1 and Snu-423 were purchased from ATCC, the other cell-lines were a gift from Cang lab of Zhejiang University. All of these cells were grown in the specified culture medium recommended by vendor and supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Total RNA from SMCC-7721, LM3, Huh7, SK-Hep1, Hep-G2, PLC, Snu-423 and LO2 cell lines were isolated using Trizol reagent (Invitrogen, USA) according to the classical procedures. RNA samples were detected the concentrations and quality by Thermo NanoDrop2000. 1 µg of total RNA was used as template RNA to synthesize first-strand cDNA using a Hifair™ III 1st Strand cDNA Synthesis Kit (Yeasen). The reverse transcript products were diluted to

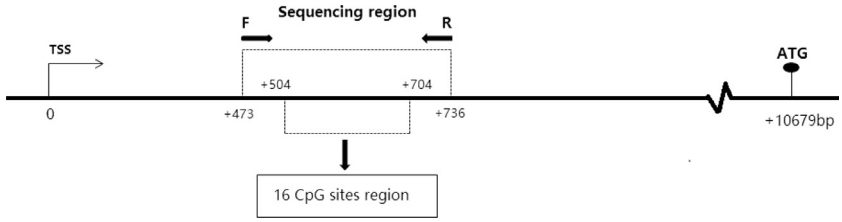

Fig. 1 Schematic diagram of CpG sites in the SPG20 promoter. The region of the CpG sites we select is indicated from +504 to +704. The sequencing region is indicated from +473 to +739 and the forward and reverse primers are shown with arrows up the diagram.

1:10, and 2ul diluted cDNA mixtures were used as template for PCR amplification using Hifair™ qPCR SYBR Green Master Mix (Yeasen). Real-time Quantitative PCR was performed with the LightCycler 480 (Roche) system. The primers for SPG20, forward primer, 5′-TGAGACCTTAGGGCTGGATG-3′ and reverse primer, 5′-CCGGGAGGACGGTTTAGAA-3′; for GAPDH, forward primer, 5′-GTGAAGCAGGCGTCGGA-3′ and reverse primer, 5′-AGCCCCAGCGTCAAAGG-3′. The $2^{-\Delta\Delta CT}$ as a calculation method was used to analyze the relative expression levels among the seven hepatoma cell lines and the non-tumour human hepatic LO2 cell line.

Sequenom analysis

The samples after bisulfite treatment were amplified by PCR, and the forward aggaagagagGGGTTTTTTTGTAGAGAAAGGTTGT reverse cagtaatacgactcactatagggagaaggctTTTAAAAAATTATTCTCAATCCCTT primers were used for Sequenom methylation analysis. The selected region located inchr13: 36919908 −36920173 (GRCh37/hg19) was the part of promoter region of SPG20 contained 16 CpG sites (Fig. 1). The detail information for CpG sites was presented in supplementary Table 1. All PCR products were through SAP cleanup, T Cleavage and Clean Resin steps before we used MassARRAY Analyzer 4 (Sequenom, USA) to analyze the quantitative methylation levels of each CpG site.

Plasmid constructs

The shRNA sequence targeted SPG20 were: forward primer, 5′- CCGGGCAAGTAGTGTTCAAGGATTTTTCAAGAGAAAATCCTTGAACACTACTTGCTTTTTTGGTACC-3′ and reverse primer, 5′- AATTGGTACCAAAAAAGCAAGTAGTGTTCAAGGATTTTCTCTTGAAAAATCCTTGAACACTACTTGC-3′. The pLKO.1-puro plasmid (Sigma SHC001) was digested with the endonucleases of AgeI and EcoRI and connected with the shRNA using T4 ligase (Thermo Fisher #EL0011) according to the manufacturer's instructions. The plasmid with shRNA was named as shSPG20 while the plasmid without shRNA was named as shControl.

Lentivirus production and infection

The shRNA expressed lentivirus were produced through co-transfecting HEK293T cells with a mixture of shSPG20 or shControl plasmid, the packaging plasmid psPAX2 (Addgene #12260), and the envelope plasmid pMD2G (Addgene #12259) at a 4:3:2 ratio using Hieff Trans™ Liposomal Transfection Reagent (YEASEN #40802ES02). We harvested the lentivirus after 48 h, and the SMMC-7721 and PLC/PRF/5 cell lines were infected by lentivirus.

Western-blot analysis

The RIPA peptide lysis buffer (Beyotime Biotechnology, Jiangsu, China) containing 1% protease inhibitors (Pierce) was used to lyse the samples and extract the protein. The protein content was quantified using the BCA kit. 20ug protein was separated by 10% SDS-PAGE gel, and transferred to PDMF membrane (Millipore) . Next, the membrane was blocked with 5% non-fat milk in TBST for 1 h at room temperature. After blocking, the membrane was incubate with SPG20 (Benchmark, #13791-1-AP) or GAPDH (Abcam, #ab181602) antibodies overnight at 4 °C. After washing, the secondary antibody against rabbit (1:10000) was used for incubation at room temperature for 1 h Blots were visualized via FDbio-Femto ECL kit (Fude biological,#FD8030).

Wound healing assay

The bottom of the 6-well plate was marked with transverse lines spaced 0.5–1 cm apart, and cells in the logarithmic growth phase were seeded and cultured overnight. When cells grew to 80– 90% confluence, a 200μL pipettor tip was used to draw scratches perpendicular to the transverse lines on bottom. The cells were washed twice in PBS and 2 ml DMEM medium containing 2%FBS was added. Then we observed the scratch and photographed under the microscope. The cells were cultured for another 48 h, and we photographed the scratch under the microscope at 24 h and 48 h. The relative widths of scratches were measured by ImageJ software.

Transwell assay

Digest the cells and the culture medium was discarded through centrifugation, washed 2 times with PBS, and resuspended in serum-free DMEM medium. Adjust cell density to $1 \times 10\text{^}6$/ml. Add 200 μl cell suspension to upper layer of a transwell chamber (Corning, #11418036) and 600 μl DMEM medium contain 10% FBS was added to lower layer. After 24 h, the chambers were removed. We wiped off cells in the

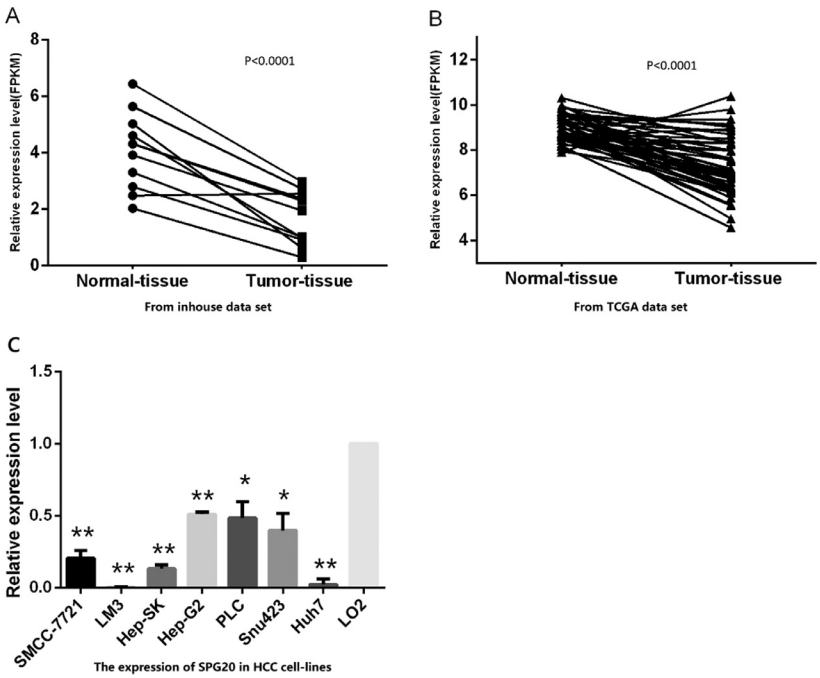

Fig. 2 A. mRNA expression level of the SPG20 gene in 11 HCC paired samples from our previous RNA sequencing study. B. Validation of SPG20 expression in TCGA database. C. The expression of SPG20 in seven HCC cell-lines related to LO2 ($^{**}p < 0.001$; $^{*}p < 0.01$).

upper chamber with a cotton swab and 4% methanol was used to fix the cells at the reverse side of chamber. Subsequently, the cells were stained with crystal violet (Beyotime, #C0121) and observed under a microscope.

Data analysis

The association analysis of individual CpG site methylation in the tumor and ANT tissues respectively was assessed using a Pearson correlation test. The paired two-tailed Student's *t*-test was used for analyzing the difference in methylation levels for each CpG site between the HCC and ANT tissues. Associations between clinic characteristics and methylation differences were assessed using multivariate logistic regression analyses. ROC curves were performed to evaluate the methylation levels of SPG20 as a biomarker for predicting the HCC metastasis. In addition, the survival curve was analyzed by Kaplan–Meier method. A P value <0.05 was statistically significant. These statistical calculations were performed using SPSS version 22.0 (IBM).

Result

Population characteristics

The clinical characteristics of the patients were listed in Table 1. Among the 160 patients, the male-to-female ratio was 131:29 and the mean of first diagnosed age was 54.0 ± 12.1 years old. In addition, 138 patients were HBV infected and 13 patients were free of liver cirrhosis. 55 patients had an alcohol habit and 17 patients had a family history of HCC. For the clinical characteristics of the tumor, 108 patients presented with incomplete fibrous capsule under the microscope. Satellite tumor tumors and microvascular invasion were detected in 15 and 31 patients respectively. 106 patients were classified as grade I refer to Union for International Cancer Control (UICC) TNM staging system.

SPG20 transcription in HCC

The expression levels of SPG20 in HCC tissues were analyzed by our previous RNA sequencing study in 11 HCC paired samples [14]. The result demonstrated that SPG20 mRNA transcription was significantly reduced in tumor tissues (Fig. 2A). In addition, we downloaded the transcriptome sequencing data of 50 paired HCC samples from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). The expression data showed that transcription levels of SPG20 were also significantly down-regulated in the tumor samples (Fig. 2B). To further confirm the population results, we also detected the expression levels of SPG20 in the different HCC cell lines by q-PCR. The expression levels of SPG20 were down-regulated in all of the seven hepatoma cell lines when compared with the normal hepatocytes LO2 cell line (Fig. 2C).

Methylation levels of individual CpG sites in HCC tissues and corresponding normal tissues

We analyzed the methylation levels of each CpG site in HCC and normal tissues. The methylation levels at CpG1 ($P<0.0001$), CpG2.3 ($P<0.0001$), CpG4 ($P<0.0001$), CpG5.6.7 ($P=0.0003$),CpG9 ($P<0.0001$),CpG10.11 ($P<0.0001$), CpG13 ($P<0.0001$), CpG14 ($P<0.0001$), and CpG15.16 ($P<0.0001$) were significantly higher in HCC

Table 2 Methylation level (%) of the detected sites in the selected region.

CpGs	Group	Mean%	⊿Mean%	p-value
CpG1	Normal	4.92	9.70	<0.0001
	Tumor	14.62		
CpG2.3	Normal	3.48	7.85	<0.0001
	Tumor	11.33		
CpG4	Normal	5.81	9.39	<0.0001
	Tumor	15.19		
CpG5.6.7	Normal	8.24	4.64	0.0003
	Tumor	12.88		
CpG9	Normal	9.38	8.67	<0.0001
	Tumor	18.05		
CpG10.11	Normal	14.23	12.75	<0.0001
	Tumor	26.98		
CpG13	Normal	5.26	11.48	<0.0001
	Tumor	16.74		
CpG14	Normal	7.85	10.84	<0.0001
	Tumor	18.69		
CpG15.16	Normal	6.08	3.97	<0.0001
	Tumor	10.04		
Mean	Normal	7.29	8.88	0.0013
	Tumor	16.18		

tissues than in normal tissues (Table 2, Fig. 3A). As for the mean methylation level of SPG20 promoter, we also found it was significantly higher in HCC tissues than that in normal tissues. The mean methylation level of SPG20 promoter was 16.2%±12.0% in HCC tissues and 7.3%±4.5% in normal tissues respectively ($P=0.0013$, Table 2). The similar results were also observed in the three selected sites which closed to the SPG20 promoter region in TCGA data set (Fig. 3B).

The correlation between the methylation levels of SPG20 and expression

We found that the hypermethylation of SPG20 promoter always implied the lower SPG20 expression. Firstly, we analyzed the correlation between methylation status and transcription levels in our previous RNA-seq data set and methylation data set. The results showed that the SPG20 transcription was significantly negatively ($P=0.0060$) related to the methylation status in the SPG20 promoter region (Fig. 3C). We further downloaded the data about the SPG20 promoter methylation levels and the corresponding transcription levels from TCGA database to analyse the correlation between methylation status and expression levels. The results were consistent with our inhouse date set ($P<0.0001$, Fig. 3D, S1 Fig).

The correlation between SPG20 promoter methylation status and clinical characteristics

We evaluated the associations between the SPG20 promoter methylation and multiple clinical features through logistic regression analyses. Among all clinicopathological parameters, we found that four CPG sites (CpG1, CpG2.3, and CpG9) and the mean methylation levels of SPG20 in tumor tissues were significantly correlated with age ($P=0.0336$, Table 3). The methylation levels of SPG20 in the HCC samples were increased in older patients. In addition, we also found that the mean methylation levels of SPG20 in normal tissues were significantly correlated with HBV infection ($P=0.0420$, Table 4). The methylation levels of SPG20 in the ANT of patients with HBV infection were higher than those without HBV infection. Meanwhile, two intra-hepatic metastasis features,

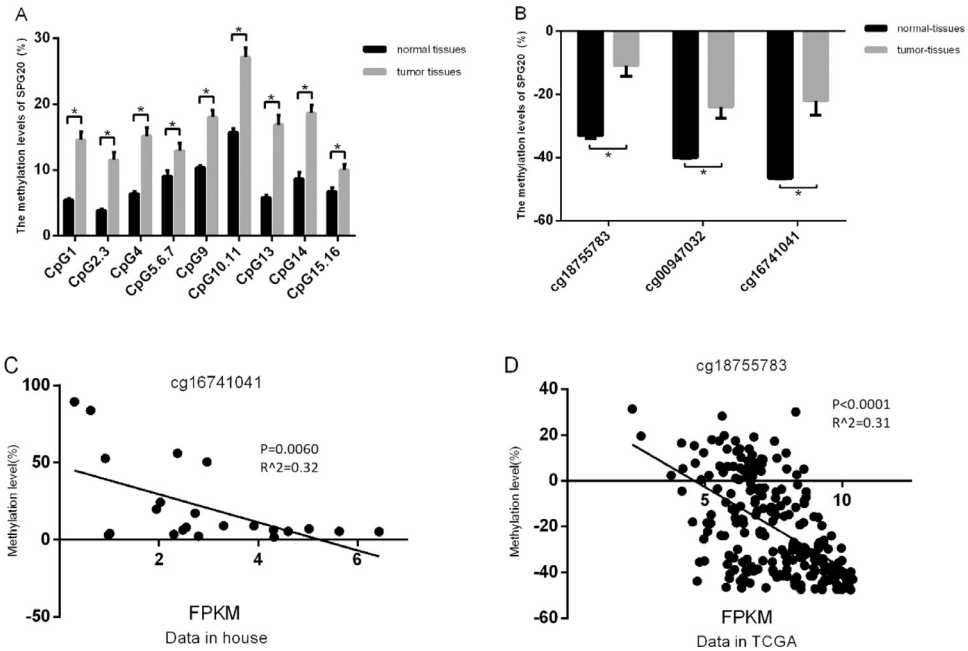

Fig. 3 A. The methylation level of all 16 CpG sites in SPG20 promoter region we detected. B. The methylation level of other 3 sites from TCGA database. C. The correlation between methylation status and expression levels from inhouse data-set. D. The correlation between methylation status and expression levels verified in TCGA database.

Table 3 The correlation betweeen the Methylation level (%) of the SPG20 and age in HCC.

Site	Items	Mean methylation level	P-value
CpG1	Age≥60	20.04%	$P=0.0016$
	Age<60	11.93%	
CpG2.3	Age≥60	15.79%	$P=0.0107$
	Age<60	9.44%	
CpG9	Age≥60	21.94%	$P=0.0062$
	Age<60	16.12%	
Mean	Age≥60	19.70%	$P=0.0085$
	Age<60	14.43%	

Table 4 The correlation between the Methylation level (%) of the SPG20 and clinical characteristics ANT.

CpG Sites	Items	Mean methylation level	P-value
	HBV infection		
CpG1	Yes	5.58%	$P=0.0366$
	No	4.52%	
CpG11	Yes	16.24%	$P=0.0224$
	No	12.52%	
Mean	Yes	8.31%	$P=0.0420$
	No	6.48%	
	Tumor number		
CpG5.6.7	1	7.92%	$P=0.0215$[a]
	>1	19.27%	
CpG14	1	7.40%	$P=0.0413$[a]
	>1	19.60%	
CpG15.16	1	6.15%	$P=0.0416$[a]
	>1	11.53%	
Mean	1	7.67%	$P=0.0183$[a]
	>1	11.33%	
	micro-satellite		
CpG5.6.7	Yes	19.93%	$P=0.0163$[a]
	No	7.93%	
CpG14	Yes	20.29%	$P=0.0344$[a]
	No	7.42%	
CpG15.16	Yes	11.79%	$P=0.0146$[a]
	No	6.16%	
Mean	Yes	11.36%	$P=0.0155$[a]
	No	7.69%	

[a] The results were calculated through multivariate logistic regression analyses, methylation of the particular CpG, age and HBV infection were entered into the model as predictors.

including micro-satellite tumors ($P=0.0009$) and tumor number ($P=0.0209$), were also found significantly associated with increased methylation levels of several CpG loci in SPG20 promotor in ANT (S4 Table). The correlations among intrahepatic metastasis features and methylation levels were remained by the logistic regesssion analyses, adjusted with age and HBV infection($P=0.0155$ and $P=0.0183$, respectively, Table 3).

Downregulated expression of SPG20 promotes HCC cell migration

We first verified the expression of SPG20 in different cell lines. The result of western-blot showed that SPG20 was highly expressed in SMCC7721, SMCC7703 and PLC/PRF/5 cell lines, while very lowly expressed in LM3, Huh7 and JHH7cell lines (Fig. 4A). Therefore, we selected SMCC7703 and PLC/PRF/5 cells for further knock-down experiment (Fig. 4B). Cell migration capacity were assessed through cell scratch test. It demonstrated that the cell migration was significantly enhanced in the SPG20 silenced cells (Fig. 4C. D). Similarly, we also detected HCC cell migration through transwell assay. The migration capacity was significantly increased in SPG20 knock-down SMCC7703 and SPG20 knock-down PLC/PRF/5 cells (Fig. 4E. F).

Discussion

In the present study, we firstly detected the methylation status of SPG20 gene promoter region in 160 paired HCC samples. Our data indicated that SPG20 was a hypermethylated gene in HCC. The levels of methylation of SPG20 in tumor tissues were significantly higher compared with ANT. Then, we analyzed the link between the expression levels of SPG20 and methylation levels. The methylation levels were negatively correlated with expression levels. In addition, we found that the methylation status was age and HBV infection related. Finally, we also found that the hypermethylation of SPG20 promoter region in ANT was significantly associated with high risk of intra-hepatic metastasis features, such as micro-satellite tumorsand tumor number. These results implied that the downregulated expression of SPG20 caused by promoter hypermethylation might play an important role in HCC initiation and progression.

DNA methylation is an important form of epigenetics alteration. More and more reliable evidence showed that the abnormal methylation status of oncogenes or tumor suppressor genes participates in tumorigenesis and can be served as a useful biomarker for early detection and prognostic evaluation [15,16]. The aberrant methylation of the SPG20 promoter has been detected in the several carcinomas such as colorectal carcinomas and renal cell carcinoma [13,17]. Besides, the downregulation of spartin expression encoded by SPG20 result to cytokinesis arrest is considered as the possible mechanism for the aneuploidy formation and genomic instability [18].

Age has proven to be associated with an accumulation of aberrations in DNA methylation in human tissues [19,20]. In nerous system, previous study has observed the association between the loss of spartin expression and aging [21]. Another recent research also indicated that the methylation alterations of SPG20 could help to discriminate childhood from adult inflammatory phenotypes in X-linked adrenoleukodystrophy [22]. In our study, we found that age, as an important risk factor for HCC, was significantly associated with the aberrant methylation of SPG20. Liver was a specific organ with a high proportion of polyploid cells. The proportion of polyploid cells in liver increased significantly with age and increased the risk for tumorigenesis [23]. This phenomenon was now believed to be due to general dysfunction of the mitotic machinery [24]. Spartin encoded by SPG20 was a crucial protein in maintaining microtubule (MT) stabilization and regulating the cytokinesis [25]. Therefore, we speculated that the increase in age might affect methylation levels of SPG20 promoter region, leading to descresed spartin expression which

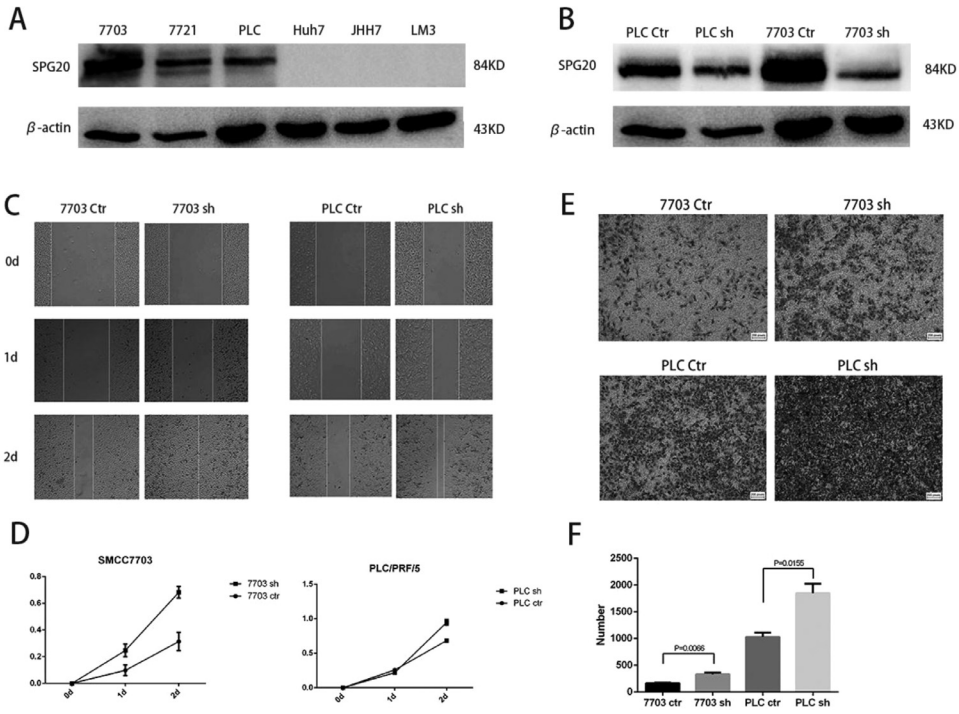

Fig. 4 A. The expression of SPG20 in different HCC cell lines; B. Verified the expression of SPG20 in knock-down HCC cell lines; C. Migration capacity of infected cells in each group; D. Quantitative analysis for the cell migration rate in each group; E. Invasion capacity of transfected cells in each group; F. Quantitative analysis for the cell Invasion number in each group.

resulted in cytokinesis arrest and increased aneuploidy proportion. It was worthy to explore in the future research.

HBV infection was a major risk factor for HCC in Chinese population. It was recognized that HBV infection could result in host chromosomal instability, which would further lead to hepatocarcinogenesis [26]. The mechanism of chromosomal instability caused by HBV infection remains unclear. In addition, increasing evidences indicated a connection between chronic inflammation caused by HBV and DNA methylation [18]. Here, we found that the methylation levels of SPG20 in ANT were positively correlated with HBV infection. Coincidentally, SPG20 played an important role in maintaining genomic stability as an essential regulator of cell division. Therefore, it was reasonable to speculate that the aberrant methylation of SPG20 caused by HBV infection might be one of the pathways leading to genomic instability. The relationship between HBV infection and SPG20 methylation in liver tissues and some unknown regulatory mechanisms was worth exploring in the future.

HCC has a poor prognosis when patients develop metastasis [27]. In our study, we showed that methylation levels of SPG20 promoter region were associated with intra-hepatic spread.The vitro experiments suggested that down-regulated expression of SPG20 could significantly improve the migration ability of HCC cells. We supposed that it mainly because of the following reasons: first, Nahm et al. [21] found the spartin encoded by SPG20 promoted microtubule instability via inhabiting bone morphogenetic protein (BMP) signaling pathway. BMP is a member of the Transforming Growth Factor β family that plays the role as a tumor suppressive signal [28]; second, previous studies implied that microtubule stabilization regulated by spartin was significant in cell cycle, signal transduction, transportation and metastasis [29] and it might be an important molecular mechanism for HCC high invasion [25].

In conclusion, we observed hypermethylation of the SPG20 promoter in HCC. Hypermethylation of the SPG20 promoter in adjacent normal tissues were correlated with high risk of intra-hepatic metastasis. In addition, the abnormal methylation of SPG20 promoter region might contribute to HCC metastasis. Besides, our results also provided a new direction in the research field of the mechanism of HCC metastasis.

Conflicts of interest

The authors have declared that no conflict of interest exists.

Author contributions

Lifeng He and Xiaoxiao Fan contributed to the data analyses and wrote the manuscript.

Xiaoxiao Fan and Lifeng He contributed to deal with Tissue Samples, DNA extraction, Bisulfite conversion and methylation.

Yirun Li and Zhaoqi Shi helped to revise this manuscript.

Bin Cui contributed to DNA bisulfite conversion.

Daizhan Zhou and Hui Lin designed the experiment, collected the samples and supervised all the process.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.04.003.

References

[1] Dutta R, Mahato RI. Recent advances in hepatocellular carcinoma therapy. Pharmacol Ther 2017;173:106–17.
[2] Forner A, Gilabert M, Bruix J, Raoul JL. Treatment of intermediate-stage hepatocellular carcinoma. Nat Rev Clin Oncol 2014;11(9):525–35.
[3] Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet 2012;13(10):679–92.
[4] Ehrlich M, Lacey M. DNA methylation and differentiation: silencing, upregulation and modulation of gene expression. Epigenomics 2013;5(5):553–68.
[5] Revill K, Wang T, Lachenmayer A, Kojima K, Harrington A, Li J, et al. Genome-wide methylation analysis and epigenetic unmasking identify tumor suppressor genes in hepatocellular carcinoma. Gastroenterology 2013;145(6):1424–35.
[6] Villanueva A, Portela A, Sayols S, Battiston C, Hoshida Y, Mendez-Gonzalez J, et al. DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. Hepatology 2015;61(6):1945–56.
[7] Manzini MC, Rajab A, Maynard TM, Mochida GH, Tan WH, Nasir R, et al. Developmental and degenerative features in a complicated spastic paraplegia. Ann Neurol 2010;67(4):516–25.
[8] Bakowska JC, Jupille H, Fatheddin P, Puertollano R, Blackstone C. Troyer syndrome protein spartin is mono-ubiquitinated and functions in EGF receptor trafficking. Mol Biol Cell 2007;18:1683–892.
[9] Hooper C, Puttamadappa SS, Loring Z, Shekhtman A, Bakowska JC. Spartin activates atrophin-1-interacting protein 4 (AIP4) E3 ubiquitin ligase and promotes ubiquitination of adipophilin on lipid droplets. BMC Biol 2010;8:72.
[10] Errico A, Claudiani P, D'Addio M, Rugarli EI. Spastin interacts with the centrosomal protein NA14, and is enriched in the spindle pole, the midbody and the distal axon. Hum Mol Genet 2004;13(18):2121–32.
[11] Zhang S, Chen Q, Liu Q, Li Y, Sun X, Hong L, et al. Hippo Signaling Suppresses Cell Ploidy and Tumorigenesis through Skp2. Cancer Cell 2017;31(5):669–84.
[12] Zhang S, Zhou K, Luo X, Li L, Tu HC, Sehgal A, et al. The polyploid state plays a tumor-suppressive role in the liver. Dev Cell 2018;44(4):447–59.
[13] Lind GE, Raiborg C, Danielsen SA, Rognum TO, Thiis-Evensen E, Hoff G, et al. SPG20, a novel biomarker for early detection of colorectal cancer, encodes a regulator of cytokinesis. Oncogene 2011;30(37):3967–78.
[14] Zhang H, Ye J, Weng X, Liu F, He L, Zhou D, et al. Comparative transcriptome analysis reveals that the extracellular matrix receptor interaction contributes to the venous metastases of hepatocellular carcinoma. Cancer Genet 2015;208(10):482–491.
[15] Nishida N, Kudo M. Alteration of epigenetic profile in human hepatocellular carcinoma and its clinical implications. Liver Cancer 2014;3(3–4):417–27.
[16] Huang CZ, Yu T, Chen QK. DNA methylation dynamics during differentiation, proliferation, and tumorigenesis in the intestinal tract. Stem Cells Dev 2015;24(23):2733–9.
[17] Slater AA, Alokail M, Gentle D, Yao M, Kovacs G, Maher ER, et al. DNA methylation profiling distinguishes histological subtypes of renal cell carcinoma. Epigenetics 2013;8(3):252–267.
[18] Comaills V, Kabeche L, Morris R, Buisson R, Yu M, Madden MW, et al. Genomic instability is induced by persistent proliferation of cells undergoing epithelial-to-mesenchymal transition. Cell Rep 2016;17(10):2632–47.
[19] Bell JT, Tsai PC, Yang TP, Pidsley R, Nisbet J, Glass D, et al. Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population. PLoS Genet 2012;8(4):e1002629.
[20] Noreen F, Roosli M, Gaj P, Pietrzak J, Weis S, Urfer P, et al. Modulation of age- and cancer-associated DNA methylation change in the healthy colon by aspirin and lifestyle. J Natl Cancer Inst 2014;106(7).
[21] Nahm M, Lee MJ, Parkinson W, Lee M, Kim H, Kim YJ, et al. Spartin regulates synaptic growth and neuronal survival by inhibiting BMP-mediated microtubule stabilization. Neuron 2013;77(4):680–95.
[22] Schluter A, Sandoval J, Fourcade S, Diaz-Lagares A, Ruiz M, Casaccia P, et al. Epigenomic signature of adrenoleukodystrophy predicts compromised oligodendrocyte differentiation. Brain Pathol 2018. [Epub]. https://doi.org/10.1111/bpa 12595.
[23] Duncan AW, Taylor MH, Hickey RD, Hanlon Newell AE, Lenzi ML, Olson SB, et al. The ploidy conveyor of mature hepatocytes as a source of genetic variation. Nature 2010;467(7316):707–10.
[24] Macedo JC, Vaz S, Bakker B, Ribeiro R, Bakker PL, Escandell JM, et al. FoxM1 repression during human aging leads to mitotic decline and aneuploidy-driven full senescence. Nat Commun 2018;9(1):2834.
[25] Chinen T, Liu P, Shioda S, Pagel J, Cerikan B, Lin TC, et al. The gamma-tubulin-specific inhibitor gatastatin reveals temporal requirements of microtubule nucleation during the cell cycle. Nat Commun 2015;6:8722.
[26] Sung WK, Zheng H, Li S, Chen R, Liu X, Li Y, et al. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet 2012;44(7):765–9.
[27] Malz M, Pinna F, Schirmacher P, Breuhahn K. Transcriptional regulators in hepatocarcinogenesis–key integrators of malignant transformation. J Hepatol 2012;57(1):186–95.
[28] Howe JR, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, et al. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 2001;28(2):184–7.
[29] Salinas PC. Modulation of the microtubule cytoskeleton: a role for a divergent canonical Wnt pathway. Trends Cell Biol 2007;17(7):333–42.