Hepatitis C virus core upregulates the methylation status of the RASSF1A promoter through regulation of SMYD3 in hilar cholangiocarcinoma cells

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Increasing evidence has been accumulated indicating the important role of epigenetic regulation in tumor genesis. Previously, we observed that the transfection of hepatitis C virus core (HCVc) protein led to malignant transformation in normal biliary cells, and that tumor suppressor gene RASSF1A was downregulated in many hilar cholangiocarcinoma patients by hypermethylation in the promoter region. In the present study, we found SET and MYND domain-containing protein 3 (SMYD3), a novel histone methyltransferase, was overexpressed in cholangiocarcinoma patients especially in those with HCV infection. Transfection of HCVc into hilar cholangiocarcinoma cell lines QBC939 and FRH0201 could upregulate the expression of SMYD3 and promote cell growth, which was consistent with the results of our clinical research. This phenomenon indicated that SMYD3 was related to the epigenetic regulation of cholangiocarcinoma genesis with HCV infection. Overexpression of SMYD3 could inhibit RASSF1A expression, whereas inhibition of SMYD3 by siRNA improved its expression. Methylation-specific polymerase chain reaction (MS-PCR) results showed the methylation status of RASSF1A promoter was regulated by SMYD3. In conclusion, HCVc could upregulate the methylation status of the RASSF1A promoter through regulation of SMYD3, and histone methylation may affect the DNA methylation of downstream gene by an unknown mechanism.

Keywords hilar cholangiocarcinoma; HCV; histone methyltransferase; DNA methylation
gene that was discovered in 2000 [15]. RASSF1A can inhibit cell proliferation, arrest cell cycle, and mediate the RAS-related apoptotic pathway [16,17]. The inactivation of RASSF1A is mainly dependent on the hypermethylation of specific promoter sequences [18]. The hypermethylation of CpG islands in the promoter area is the main mechanism of inactivation of RASSF1A gene in extrahepatic cholangiocarcinoma [19].

In preliminary experiments, we found that the promoter of RASSF1A gene was not methylated in human normal bile duct epithelial cells (BEC) that did not express SMYD3, but it was partially methylated in human hilar cholangiocarcinoma cell line (QBC939) expressing SMYD3, which indicated that the expression of RASSF1A promoter may be related to SMYD3. Moreover, RASSF1A has the sequence 5’-GAGGGG-3’ in its promoter region, which is exactly the SMYD3 binding motif. It was speculated that the expression of SMYD3 may promote the methylation of the promoter of RASSF1A. Thus, this study was designed to determine the correlation between HCVc and SMYD3, and the methylation status of the downstream tumor suppressor gene RASSF1A in hilar cholangiocarcinoma cells.

Materials and Methods

Materials and reagents
The plasmid pcDNA3.1-HCVc (573 bp, genotype 1b) was kindly provided by Prof. Jun Cheng (Ditan Hospital, Beijing, China). Plasmid pGPU6/green fluorescent protein (GFP)/Neo, T-easy vector, and pAAV vector were kindly provided by Prof. Shangwu Wang (Sun yat-sen University, Guangzhou, China). The human hilar cholangiocarcinoma cell line QBC939 was kindly provided by Prof. Shuguang Wang (The Third Military Medical University, Chongqing, China), and the human hilar cholangiocarcinoma cell line FRH0201 was kindly provided by Prof. Xiaopeng Wu (Shandong University, Jinan, China). DNA Gel Extraction Kit and RIPA buffer was obtained from Beyotime (Shanghai, China). Streptavidin–biotin complex (SABC) immunohistochemistry kit was purchased from Boster (Wuhan, China). Cell culture medium and fetal bovine serum were purchased from Gibco (Carlsbad, USA). The siRNA targeting human SMYD3, the negative control siRNA, and the 5-(and-6)-carboxyfluorescein-labeled siRNA were purchased from GenePharma (Shanghai, China). Transfection-selective drug G418 was purchased from Gibco (Carlsbad, USA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Pulilai (Beijing, China). Antibodies against human HCVc and SMYD3 were purchased from Abcam (Cambridge, UK). The transfection reagent Effectene was purchased from Ferments (Burlington, Canada). DNA endonuclease and ligase and T4 ligase following the instructions. The HCVc fragment was digested with DNA endonuclease from pcDNA3.1-HCVc and ligated to the plasmid pGPU6/GFP/Neo by endonuclease and T4 ligase following the instructions. Transfection reagent Effectene was purchased from Ferments (Burlington, Canada).

Plasmid construction
To obtain the fluorescent plasmid for flow cytometry and checking transfection efficiency, plasmid pGPU6/GFP/Neo-HCVc was constructed. The HCVc fragment was digested with DNA endonuclease from pcDNA3.1-HCVc and ligated to the plasmid pGPU6/GFP/Neo by endonuclease and T4 ligase following the instructions. Electrophoresis and gene sequencing were performed to confirm that HCVc fragment was inserted successfully to the plasmid.

The sequence of SMYD3 (NCBI reference No: NM_022743.1) was retrieved from human cholangiocarcinoma tissue. The PCR primers to amplify SMYD3 were as following: 5’-CCGCTCGAGATGGAGCCGCTGAAGGTG-3’ (forward), 5’-CCGGAATTCTTAGGCTGCTCTGATGTTGGCG-3’ (reverse). Then fragment was ligated to the plasmid pEGFP-C3 by T4 ligase to construct the plasmid pEGFP-SMYD3 following the instructions and gene

plasmid extract kit I was purchased from Tiangen (Beijing, China).

Tissue samples
Twenty-two bile duct tissue samples were collected from 22 patients in our hospital from 2005 to 2010. In these cases, seven samples were diagnosed as hilar cholangiocarcinoma with HCV infection, eight samples were diagnosed as hilar cholangiocarcinoma without HCV infection, and other seven samples were normal bile duct tissues (control group). The ethical approval was obtained from our hospital review board and all patients were informed and agreed to participate in our research.

Immunohistochemistry staining
Tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemical staining was carried out using the SABC immunohistochemistry kit. One paraffin-embedded block of tissue was selected from each case and cut into 4 μm sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide before conducting antigen retrieval using a microwave oven at 95°C for 5 min, and cooling at room temperature for 10 min for two times. After washing with phosphate-buffered saline (PBS), 5% bovine serum albumin was applied for 10 min. The sections were incubated with anti-SMYD3 antibody (1:75) overnight at 4°C. After washing by PBS, a biotin-conjugated secondary antibody was applied for 20 min followed by SABC treatment for an additional 20 min. DAB chromogenic agent kit (Boster) was used to develop color and the samples were counterstained with hematoxylin. SMYD3 protein expression was then observed with microscope.

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sequencing was performed to confirm that SMYD3 fragment was inserted correctly.

Cell transfection
The plasmid pGPU6/GFP/Neo-HCVc was, respectively, transfected into the FRH0201 and QBC939 cells with Effectene reagent. After 24 h of transfection, the media were changed and G418 was added in each well to a final concentration of 100 μg/ml to screen the monoclonal strain. The monoclonal strain was cultured for 1 month and then transferred into a separate six-well plate containing G418 (250 μg/ml for QBC939 and 200 μg/ml for FRH0201). Cells transfected with empty pGPU6/GFP/Neo plasmid were treated as empty control. The expression of HCVc in the cell lines was analyzed by western blot.

To observe the relationship between SMYD3 and RASSF1A, the plasmid pEGFP-SMYD3 was transiently transfected into the cholangiocarcinoma cell lines to make an overexpression of SMYD3. siRNA (5'-AACATCTACC AGCTGAAGGTG-3') targeting SMYD3 was transfected to inhibit SMYD3 expression. Effectene (Qiagen) was used as the transfection reagent. Then, the transfected cells were incubated for 24 h, and the transfection efficiency was examined by flow cytometry.

CCK-8 assay
Each of the two kinds of cells was divided into three groups: HCVc plasmid transfected group, empty plasmid transfected group, and untransfected group. The cells were cultured in 96-well plates at an initial density of 1×10⁴ cells/well in 200 μl of growth medium (3 wells for each group) and incubated at 37°C. Once the cells grew into the logarithmic growth phase (pre-experiment showed FRH0201 started at the 11th hour and QBC939 at 14th hour), 20 μl of CCK8 (Dojindo, Kumamoto, Japan) was added. After incubating for 2 h, the absorbance at 630 nm was detected with a MK3 ELISA reader (Labsystem Dragon, Vantaa, Finland) every 2 h.

Western blot
Total protein was extracted using RIPA buffer with protease inhibitor. The BCA protein assay kit (Beyotime) was used to determine the concentration of protein. Equal amounts (20 μg) of total protein were loaded onto a 10% SDS polyacrylamide gel, and then subsequently transferred to a polyvinylidene fluoride (PVDF) membrane via electrophoresis. The membranes were blocked with 5% nonfat milk in tris-HCl buffer and tween (TBST) for 2 h at room temperature and incubated with anti-SMYD3 antibody at the dilution of 1:500 overnight at 4°C. After washing with TBST buffer for three times (10 min each time), the PVDF membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1:4000) for 2 h at room temperature. ECL detection reagent was used to detect the bands. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control. Image analysis was performed by IMAGEJ plus.

Quantitative real-time PCR
After transfection for 24 h, RNA was extracted by RNAiso plus (TaKaRa, Dalian, China) and the RT reaction was

**Figure 1** Immunohistochemistry result of SMYD3 expression in cholangiocarcinoma patients The SMYD3 expression is nearly negative in normal bile ducts (A), and is much stronger in cholangiocarcinoma patients with HCV infection (C) than those cholangiocarcinoma patients without HCV infection (B). Bar = 50 μm.
performed using TaKaRa RT reagent kit. The products were run on a Roche LC480 instrument with the SYBR Premix Ex Taq. The reaction was performed as described by the manufacturer protocol. A negative control without cDNA was used to detect possible contamination. The Q-PCR reaction proceeded as follows: 95°C for 30 s, then 30 cycles including 90°C for 30 s, 60°C for RASSF1A/58°C (for β-actin) for 30 s, and 72°C for 1 min. Total RNA from normal biliary duct tissue was used as a control. The result was checked by 2% agarose gel electrophoresis.

Methylation-specific polymerase chain reaction (MS-PCR)

The methylation status in the RASSF1A promoter region was detected by MS-PCR using MethylDetector Kit (Active-Motif, Carlsbad, USA) according to the manufacturer’s protocol. To amplify the bisulfate-converted RASSF1A promoter sequences, the methylated (M) and unmethylated (U) primers were designed according to our previously published protocol [20] and were listed as follows: RASSF1A-M (sense): 5′-GGGTCTTGGAGAGCGG-3′; RASSF1A-M (anti-sense): 5′-GCTAACAAAACGGCGAAACCG-3′; RASSF1A-U (sense): 5′-GGTTTTTGTAGAGCTGTGTGTAG-3′; and RASSF1A-U (anti-sense): 5′-CCTAAACAAAAACACAAAACACCTGGG-3′. The annealing temperature for RASSF1A-M primer is 64°C and for RASSF1A-U primer is 59°C. Both the products’ length is 169 bp.

Results

SMYD3 expression in cholangiocarcinoma patients

The results showed that the SMYD3 positive expression rate was 0 (zero of seven) in the normal bile ducts [Fig. 1(A)], 25% (two of eight) in the hilar cholangiocarcinoma tissues without HCV infection [Fig. 1(B)], and 85.71% (six of seven) in the hilar cholangiocarcinoma tissues with HCV infection [Fig. 1(C)]. This phenomenon indicated that SMYD3 might express highly in the hilar cholangiocarcinoma tissues.

RT–PCR

The total RNA of the cells was extracted using RNAiso plus and RT–PCR was performed using RNA-PCR kit (Takara) following the manuals. The primers of RASSF1A (280 bp) were as following: sense, 5′-GGGTTTTGCGAGAGCGG-3′; anti-sense, 5′-GGTTTTGCGAGAGCGG-3′; β-Actin (forward, 5′-TGGCACAAGCAGACGCTCTGAT-3′; reverse, 5′-ACACGCGCCATATTTCCTCT-3′). The PCR reaction was proceeded as follows: 95°C for 30 s, then 30 cycles including 90°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Total RNA from normal biliary duct tissue was used as a control. The result was checked by 2% agarose gel electrophoresis.

Figure 3 Determination of cells growth curve of FRH0201 cells (A) and QBC939 cells (B) after HCV transfection by CCK-8 assay *P < 0.05 compared with untransfected or empty plasmid transfected group.

Figure 4 Detection of SMYD3 expression after HCV plasmid transfection Western blot analysis was performed to detect SMYD3 expression after transfection of SMYD3 in the cholangiocarcinoma cell lines FRH0201 and QBC939. The expression of SMYD3 is upregulated by 89.31% in FRH0201 and 98.12% in QBC939 cells in the HCV transfected group compared with the empty plasmid transfected group or the no treatment group. GAPDH was used as a control.

TTTGCCTGGG-3′ (anti-sense). The annealing temperature for RASSF1A was 60°C and for β-actin was 58°C. The PCR reaction was proceeded as follows: 95°C for 30 s, then 30 cycles including 95°C for 30 s, 60°C (for RASSF1A)/58°C (for β-actin) for 30 s, and 72°C for 1 min. Total RNA from normal biliary duct tissue was used as a control. The result was checked by 2% agarose gel electrophoresis.
Generation of stably transfected cell lines
The electrophoresis image showed the HCVc was correctly inserted into the pGPU6/GFP/Neo plasmid [Fig. 2(A)]. Sequencing result showed that the direction of transcription was correct. Western blot showed that HCVc protein expression was found in HCVc plasmid transfected group, not in the empty group or the untreated groups [Fig. 2(B)].

HCV transfection promoted SMYD3 expression
The western blot for SMYD3 expression is shown in Fig. 4. The expression of SMYD3 was upregulated by 89.31% in FRH0201 cells and by 98.12% in QBC939 cells after HCVc transfection, which was higher than those in the empty group and untransfected group.

Measurement of cell growth
Figure 3(A) showed the growth curve of FRH0201 cells transfected with HCVc plasmid, empty plasmid, and the untransfected cell. Figure 3(B) represents the growth curve of QBC939. SPSS 16.0 was used for variance analysis. As suggested in the results, the transfected FRH0201-HCVc and QBC939-HCVc cell lines grew faster than the non-transfected cell lines \( P < 0.05 \), but no significant difference for the growth was observed between empty plasmid transfected group and untransfected group in FRH0201 and QBC939 cells.

Upregulation of SMYD expression by pEGFP-SMYD3 transfection
The western blot for SMYD3 expression is shown in Fig. 5. The expression of SMYD3 was upregulated by 95.16% in FRH0201 cells, and by 75.56% in QBC939 cells compared with the untransfected cells, respectively [Fig. 5(B)]. Western blot showed that the level of SMYD3 protein was upregulated by 90.72% in FRH0201 cells and by 67.83% in QBC939 cells compared with the untransfected cells [Fig. 5(C)].
Downregulation of SMYD3 expression by siRNA transfection

The siRNA transfection efficiency was 89.98% in FRH0201 cells and 85.23% in QBC939 cells. Real-time PCR results showed that the expression of SMYD3 mRNA was downregulated by 98.23% ± 1.47% in FRH0201 cells, and downregulated by 82.18% ± 2.16% in QBC939 cells compared with the untransfected cells. The level of SMYD3 protein was downregulated by 88.71% in FRH0201 cells and 47.83% in QBC939 cells compared with the untransfected cells.

Detection of RASSF1A expression after SMYD3 gene knockdown

RT–PCR results showed that the expression of RASSF1A in both of hilar cholangiocarcinoma cell lines was much lower (32.73% for FRH0201 and 47.63% for QBC939) than in the normal biliary cells. SMYD3 knockdown resulted in the upregulation of RASSF1A in FRH0201 (87.52%) and QBC939 (61.81%) cells. On the contrary, the overexpression of SMYD3 significantly inhibited the expression of RASSF1A.

Detection of the methylation situation of RASSF1A promoter by MS-PCR

As shown in Fig. 8, histone methyltransferase SMYD3 could affect the methylation situation of RASSF1A. Overexpression of SMYD3 could increase DNA methylation level, whereas SMYD3 inhibition caused it to decrease. This phenomenon indicated that SMYD3 expression was possibly associated with the methylation level of RASSF1A promoter by some unknown mechanism.

Discussion

According to the immunohistochemistry results of the clinical samples, we speculated that SMYD3 was correlated with HCVc in hilar cholangiocarcinoma cell lines. Our experiments in vitro provided more evidence of the relationship between HCVc and SMYD3. In our study,
we found that the growth speed of the hilar cholangiocarcinoma cell lines was improved, and the expression of SMYD3 was increased after HCVc transfection in cholangiocarcinoma cell lines QBC939 and FRH0201. This phenomenon indicated that the expression of SMYD3 was related to HCVc transfection, and might play an important role in the tumor genesis of hilar cholangiocarcinoma. We speculated that HCVc may be the initiation factor of SMYD3, whereas SMYD3 might relate to either activation of some oncogene or inactivation of tumor suppressor gene.

Previously we found that the promoter of tumor suppress gene RASSF1A was methylated in 58.33% cases of hilar cholangiocarcinoma patients [21]. The application of the DNA methyltransferase inhibitor 5-N-2’-deoxycytidine on the bile duct cell line can induce re-expression of this gene and inhibit cholangiocarcinoma cell growth [22]. As we know, DNA methylation, especially promoter region CpG island hypermethylation, is a common cause of inactivation for many tumor suppressor genes [23], and the inactivation of RASSF1A is mainly through this mechanism [24]. We deduced the inactivation of RASSF1A was also resulted by epigenetic modification of hypermethylation in hilar cholangiocarcinoma.

In this study, we found that the expression of RASSF1A was lower in the cholangiocarcinoma cells than in normal biliary duct cells. The expression of RASSF1A was increased when siRNA was used to disturb the expression of SMYD3, whereas it was decreased by overexpression of SMYD3. Then MS-PCR was performed to check the RASSF1A gene promoter methylation status. The results showed that the methylation status of the RASSF1A gene promoter in the hilar cholangiocarcinoma cell lines QBC939 and FRH0201 was positively correlated with the expression levels of SMYD3. Thus, we speculated that SMYD3 could affect the methylation situation of RASSF1A promoter, and regulate the expression of RASSF1A.

Both histone methylation and DNA methylation are very important for regulating the expression of multiple genes in many types of tumors. However, only in recent years, the links between the two processes have been gradually revealed. In 2001, Tamaru and Selker found that the mutation of both of the widely distributed and conserved Su(var)-like methyltransferase and histone H3K9 tyrosine can lead to the loss of DNA methylation, implying that histone methylation may be a prerequisite for DNA methylation [25]. The results of our study showed that the methylation situation of RASSF1A was positively related to the expression of SMYD3.

In conclusion, epigenetic changes in hilar cholangiocarcinoma cell line suggested that HCVc infection increases the risks of hilar cholangiocarcinoma by promoting the methylation of the promoter of RASSF1A through upregulation of HMTase SMYD3. Epigenetic regulation plays an important role in the process of the HCVc–SMYD3–RASSF1A pathway in hilar cholangiocarcinoma, and histone methylation may affect DNA methylation of the downstream gene. However, the specific mechanism still needs further investigation.

**Acknowledgements**

We are very grateful to all colleagues at the Department of General Surgery and Department of Oncology of Sun Yat-sen Memorial Hospital of Sun Yat-sen University for their cooperation in our experiments.
**Funding**

This work was supported by a grant from the National Natural Science Foundation of China (30872485).

**References**


Acta Biochim Biophys Sin (2011) | Volume 43 | Issue 5 | Page 361