Research Article

Promoter Methylation of SFRP3 Is Frequent in Hepatocellular Carcinoma

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Oncogenic activation of the Wnt/β-catenin signaling pathway is common in human cancers. The secreted frizzled-related proteins (SFRPs) function as negative regulators of Wnt signaling and have important implications in carcinogenesis. Because there have been no reports about the role of SFRP3 in hepatocellular carcinoma (HCC), we investigated the level of methylation and transcription of SFRP3. Four HCC cell lines, 60 HCCs, 23 cirrhosis livers, 37 chronic hepatitis livers, and 30 control livers were prescreened for SFRP3 promoter methylation by methylation-specific polymerase chain reaction (MS-PCR) and bisulfite sequencing. SFRP3 promoter methylation was observed in 100%, 60%, 39.1%, 16.2%, and 0% in HCC cell lines, primary HCCs, cirrhosis livers, chronic hepatitis livers, and control livers, respectively. Demethylation treatment with 5-aza-2'-deoxycytidine in HCC cells restored or increased the SFRP3 mRNA expression. We next used quantitative MS-PCR (QMSP) to analyze the methylation level of SFRP3 in 60 HCCs and their corresponding nontumor tissues. Methylation of SFRP3 promoter region in HCCs increased significantly compared with control tissues. There is a positive correlation between promoter hypermethylation and SFRP3 mRNA downregulation. Our data suggest that promoter hypermethylation of SFRP3 is a common event in HCCs and plays an important role in regulation of SFRP3 mRNA expression.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and accounts for as many as 1 million deaths annually worldwide [1–5]. The major risk factors include chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, environmental carcinogens such as aflatoxin B1 (AFB1), alcoholic cirrhosis, and inherited genetic disorder such as hemochromatosis, Wilson disease, and tyrosinemia. Among them, HBV, HCV, and AFB1 are responsible for approximately 80% of all HCC [1, 2]. Research on molecular genetics and pathogenesis of HCC has become a hot spot in cancer study because of its scientific merits and its clinical importance.

Despite rapid expansion of information obtained from these researchers, the molecular mechanism of hepatocarcinogenesis and molecular genetics of HCC remain elusive. The Wnt/β-catenin signaling pathway plays an important role in liver physiology and pathology by regulating a variety of crucial cellular events, including differentiation, proliferation, and survival [6–8]. The Wnt/β-catenin pathway can be activated through mutations in CTNNB1 (encoding β-catenin), AXIN1, and AXIN2 [6, 9] in human HCC. The common event is the stabilization of β-catenin, which translocates into the nucleus and associates with the T-cell factor (TCF) family of transcription factors for efficient activation of Wnt target genes [10–17]. In addition to genetic mutations,
epigenetic changes are also involved in the aberrant activation of Wnt/β-catenin signaling pathway in cancer cells [6, 9, 18–22].

Abnormal hypermethylation of CpG islands serves as another mechanism for inactivation of the tumor suppressor gene (TSG) in cancer [23–25]. Hypermethylation of gene promoters has been demonstrated as an early event in hepatocellular carcinogenesis [26–28]. The secreted frizzled-related proteins (SFRPs) function as negative regulators of Wnt signaling and have important implications for carcinogenesis [29]. The secreted frizzled-related protein (SFRP) family plays a significant role in the inhibition of the Wnt signaling pathway in various cancers [30]. The frizzled-related protein (SFRP3) is generally thought to be an inhibitor of Wnt signaling in several cancers [31, 32]. Some reports have demonstrated that SFRP3 has tumor-suppressing activities and could inhibit cell invasiveness in prostate cancer and melanoma cells [31, 32]. However, SFRP3 promotes cell growth, invasion, and inhibition of apoptosis in renal cancer cells [33]. Because there have been no reports about the role of SFRP3 in hepatocellular carcinoma (HCC), we investigated the level of methylation and transcription of SFRP3.

Recently, we have shown that SFRPs are often downregulated through promoter hypermethylation in HCC cell lines and clinical HCC tissues [18, 34]. Furthermore, we have demonstrated that restoration of SFRP3 could attenuate Wnt signaling in HCC cells with β-catenin mutation, decrease aberrant accumulation of free β-catenin in the nucleus, and then suppress cell growth [34]. We hypothesized that CpG island methylation of the SFRP3 promoter may play an important role in regulating SFRP3 expression in HCC. To test this hypothesis, we used MS-PCR, QMSP, and bisulfite sequencing method to analyze the SFRP3 methylation pattern in HCCs. The mRNA expression was assessed by quantitative RT-PCR assay. Further, we determined whether treatment of HCC cell lines with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-Aza-CdR), could then restore or increase expression of the SFRP3 mRNA.

2. Materials and Methods

2.1. Tissue Specimens. Sixty paired HCC samples (including HCC tissues, DNA, and RNA samples) and 30 hepatic hemangioma tissues were provided by the Taiwan Liver Cancer Network (TLCN). The TLCN is funded by the National Science Council to provide researchers in Taiwan with primary liver cancer tissues and their associated clinical information. The diagnosis of HCC was confirmed by histology. Experienced pathologist classified the nontumor tissues as chronic hepatitis livers (23 cases) and cirrhosis livers (37 cases). The use of the 60 HCC tissues, paired nontumor parts, and 30 hepatic hemangioma tissues (as control livers) in this study was approved by the Institutional Review Board and the TLCN User Committee.

2.2. Cell Lines. We obtained three human HCC cell lines from the American Type Culture Collection (ATCC, Rockville, MD): HepG2, HA22T, Hep3B, and TONG. They were all grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (w/v) fetal bovine serum, penicillin at 100 U/mL, streptomycin at 100 μg/mL, and L-glutamine at 2 mmol/L (all from Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% (v/v) CO₂ in air.

2.3. 5-Aza-2'-deoxycytidine Treatment. HCC cells were seeded at a density of 1 × 10⁵ cells/100-millimeter dish and allowed to attach for 24 hr. Cells were incubated in 5 μM 5-aza-2'-deoxycytidine (5-Aza-CdR, Sigma Chemical Co., St. Louis, MO) diluted in phosphate-buffered saline (PBS) or in PBS alone for 96 hr to analyze the effect of methylation inhibition on SFRP3 mRNA expression. All incubations were performed in duplicate dishes, and cells were harvested directly for RNA and DNA isolation.

2.4. DNA Extraction. Genomic DNA was extracted from cell lines and tissue samples using a commercial DNA extraction kit (QiAmp Tissue Kit; Qiagen, Hilden, Germany). DNA was isolated according to the manufacturer’s protocol.

2.5. Bisulfite Modification and Methylation-Specific PCR (MS-PCR). Genomic DNA isolated from cells and tissue was subjected to bisulfite methylation analysis. We treated DNA with bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA) according to the protocol described in the user manual. Briefly, one μg of genomic DNA was denatured by incubation with 0.2 M NaOH. Aliquots of 10 mM hydroquinone and 3 M sodium bisulfite (pH 5.0) were added and the solution was incubated at 50°C for 16 hr. Treated DNA was purified on a Zymo-Spin I column, desulfonated with 0.3 M NaOH, repurified on a Zymo-Spin I column, and resuspended in 20 μL elution buffer. MS-PCR [35] was carried out in a volume of 25 μL containing 1 μL of the sodium-bisulfite-treated DNA with Gold Taq DNA polymerase (PE Applied Biosystems, Foster City, CA) as follows. After heating at 92°C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 92°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 30 sec, followed by a final 10 min extension at 72°C. The PCR products were analyzed by electrophoresis on a 3% agarose gel. The experiments were repeated three times to ensure reproducibility. The sequences of SFRP3 promoter, primer, and probes are summarized in Table 1.

2.6. Bisulfite Sequencing. Bisulfite–treated genomic DNA was amplified using specific primers for human SFRP3. Amplified PCR product was purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequencing was performed on at least 5 individual clones using the 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The primer sequences and the locations are summarized in Table 1.

2.7. Quantitative Methylation-Specific PCR (QMSP). TagMan-based QMSP (MethylLight) [36] method was used to determine the methylation level of HCCs. We used type
II collagen gene (COL2A) for an internal reference gene by amplifying the non-CpG sequences. Each sample was analyzed three times. The genomic DNA treatment with M.Sss I methyltransferase (New England Biolabs, Beverly, MA) was used as positive control. The QMSP reactions were done as our previous report [37]. The relative DNA methylation was determined based on the threshold cycles (Ct) of the gene of interest and of the internal reference gene (COL2A). The relative DNA methylation level [sample_gene/sample.COL2A] was calculated by the ΔCt method [36, 38]. Testing results with Ct-value of COL2A greater than 40 were determined as detection failure.

2.8. Quantitative RT-PCR. Quantitative RT-PCR analysis was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Forster City, USA). The match primers and TaqMan Probe were obtained from commercial Applied Biosystems Tagman Assay-on-Demand Gene Expression products. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control. PCR reaction was carried out using TaqMan PCR master mix reagents kit. Relative gene expression was determined based on the threshold cycles (Ct) of the gene of interest and of the internal reference gene. The mRNA levels of the interest genes were expressed as the ratio of the interest gene to GAPDH mRNA for each sample. The level of each interest gene mRNA in each cancer was compared to the level in the corresponding nontumor part [39]. The average Ct value of the GAPDH gene was subtracted from the average Ct value of the interest genes for each sample: SFRP3 ΔCt = (Avg. SFRP3 Ct – Avg. GAPDH Ct) and SFRP3 ΔΔCt = (Avg. SFRP3 ΔCtumor – Avg. SFRP3 ΔCtnontumor). The fold change (2−ΔΔCt) in expression of the target genes (SFRP3) relative to the internal control gene (GAPDH) of each analyzed HCC sample was calculated [18, 39].

2.9. Statistical Analysis. Associations between methylation of SFRP3 and clinical parameters were analyzed by using a chi-square test and Fisher’s exact test, where necessary. We correlated the SFRP3 methylation status with the liver disease status (control, chronic hepatitis, cirrhosis liver, and HCC) and downregulation of SFRP3 mRNA expression. Significant differences were analyzed using the paired sample t-test or Mann-Whitney U test. The significance level was defined as P value < 0.05.

3. Results

3.1. Hypermethylation of SFRP3 Promoter in Primary HCCs. To investigate the promoter methylation of SFRP3 in HCC, we first tested for promoter methylation in 30 control livers, 60 primary HCCs, and their corresponding nontumor tissues using MSP (Figures 1a and 1b, Table 2). Aberrant promoter methylation of SFRP3 gene was observed in 60%, 39.1%, 16.2%, and 0% in primary HCCs, cirrhosis livers, and normal controls, respectively.

Sequence of SFRP3 promoter:

| Primer name | Assay |
|-------------|-------|
| SFRP3 UF    | MSPCR |
| SFRP3 UR    | MSPCR |
| BS01 F      | BS sequencing |
| BS01 R      | BS sequencing |
| SFRP3 MF    | MSPCR, QMSP |
| SFRP3 MR    | MSPCR, QMSP |
| SFRP3 F     | QRT-PCR (SyBr) |
| SFRP3 R     | QRT-PCR |

Table 1: The primer and probe sequences used in this study.

**II collagen gene (COL2A) for an internal reference gene by amplifying the non-CpG sequences. Each sample was analyzed three times. The genomic DNA treatment with M.Sss I methyltransferase (New England Biolabs, Beverly, MA) was used as positive control. The QMSP reactions were done as our previous report [37]. The relative DNA methylation was determined based on the threshold cycles (Ct) of the gene of interest and of the internal reference gene (COL2A). The relative DNA methylation level [sample_gene/sample.COL2A] was calculated by the ΔCt method [36, 38]. Testing results with Ct-value of COL2A greater than 40 were determined as detection failure.**
Table 2: SFRP3 mRNA expression in primary HCCs by relative quantitative RT-PCR.

| Patient no. | SFRP3 methylation | \( \Delta Ct \)  | \( \Delta \Delta Ct \)  | SFRP3 tumor part | \( \Delta Ct \) tumor – \( \Delta Ct \) nontumor | Rel. to nontumor |
|-------------|-------------------|-----------------|---------------------|-----------------|------------------------------------------|------------------|
| 1T          | U                 | 9.03            | 1.68                | 0.312           |                                          |                  |
| 1NT         | U                 | 7.35            |                     |                 |                                          |                  |
| 2T          | M                 | 10.05           | 2.81                | 0.1426          |                                          |                  |
| 2NT         | M                 | 7.24            |                     |                 |                                          |                  |
| 3T          | U                 | 7.63            | −0.47               | 1.3851          |                                          |                  |
| 3NT         | U                 | 8.10            |                     |                 |                                          |                  |
| 4T          | U                 | 7.58            | −0.51               | 1.4191          |                                          |                  |
| 4NT         | U                 | 8.09            |                     |                 |                                          |                  |
| 5T          | U                 | 11.54           | 0.82                | 0.5684          |                                          |                  |
| 5NT         | U                 | 10.72           |                     |                 |                                          |                  |
| 6T          | U                 | 5.92            | −0.29               | 1.2226          |                                          |                  |
| 6NT         | U                 | 6.21            |                     |                 |                                          |                  |
| 7T          | M                 | 7.40            | 1.38                | 0.3856          |                                          |                  |
| 7NT         | U                 | 6.03            |                     |                 |                                          |                  |
| 8T          | U                 | 15.00           | 6.10                | 0.0146          |                                          |                  |
| 8NT         | U                 | 8.91            |                     |                 |                                          |                  |
| 9T          | M                 | 8.95            | 1.91                | 0.2661          |                                          |                  |
| 9NT         | U                 | 7.04            |                     |                 |                                          |                  |
| 10T         | M                 | 9.03            | 1.79                | 0.2892          |                                          |                  |
| 10NT        | M                 | 7.24            |                     |                 |                                          |                  |
| 11T         | M                 | 15.00           | 9.03                | 0.0019          |                                          |                  |
| 11NT        | M                 | 5.97            |                     |                 |                                          |                  |
| 12T         | M                 | 9.30            | 1.35                | 0.3923          |                                          |                  |
| 12NT        | M                 | 7.75            |                     |                 |                                          |                  |
| 13T         | U                 | 9.62            | 1.58                | 0.3356          |                                          |                  |
| 13NT        | U                 | 8.04            |                     |                 |                                          |                  |
| 14T         | U                 | 6.27            | −0.71               | 1.6358          |                                          |                  |
| 14NT        | U                 | 6.98            |                     |                 |                                          |                  |
| 15T         | M                 | 15.00           | 7.90                | 0.0042          |                                          |                  |
| 15NT        | U                 | 7.10            |                     |                 |                                          |                  |
| 16T         | M                 | 15.00           | 7.14                | 0.0071          |                                          |                  |
| 16NT        | M                 | 7.86            |                     |                 |                                          |                  |
| 17T         | M                 | 9.34            | 1.13                | 0.4569          |                                          |                  |
| 17NT        | M                 | 8.21            |                     |                 |                                          |                  |
| 18T         | U                 | 5.30            | −1.01               | 2.0069          |                                          |                  |
| 18NT        | U                 | 6.11            |                     |                 |                                          |                  |
| 19T         | M                 | 6.75            | 1.04                | 0.4863          |                                          |                  |
| 19NT        | M                 | 5.71            |                     |                 |                                          |                  |
| 20T         | U                 | 15.00           | 7.87                | 0.0043          |                                          |                  |
| 20NT        | U                 | 7.14            |                     |                 |                                          |                  |
| 21T         | M                 | 15.00           | 8.13                | 0.0036          |                                          |                  |
| 21NT        | U                 | 6.87            |                     |                 |                                          |                  |
| 22T         | U                 | 9.92            | 3.48                | 0.0899          |                                          |                  |
| 22NT        | U                 | 6.45            |                     |                 |                                          |                  |
| 23T         | M                 | 9.05            | 1.42                | 0.3737          |                                          |                  |
| 23NT        | M                 | 7.63            |                     |                 |                                          |                  |
Table 2: Continued.

| Patient no. | SFRP3 methylation | ΔCt SFRP3-GAPDH | ΔΔCt | ΔCt tumor − ΔCt nontumor | SFRP3 tumor part Rel. to nontumor |
|-------------|--------------------|------------------|------|---------------------------|----------------------------------|
| 24T         | M                  | 8.47             | 1.24 | 0.4248                    |                                  |
| 24NT        | M                  | 7.23             |      |                           |                                  |
| 25T         | M                  | 6.96             | 0.61 | 0.6552                    |                                  |
| 25NT        | M                  | 6.35             |      |                           |                                  |
| 26T         | U                  | 5.14             | 0.11 | 0.9298                    |                                  |
| 26NT        | U                  | 5.04             |      |                           |                                  |
| 27T         | M                  | 12.37            | 5.31 | 0.0253                    |                                  |
| 27NT        | M                  | 7.06             |      |                           |                                  |
| 28T         | M                  | 15.00            | 6.21 | 0.0136                    |                                  |
| 28NT        | U                  | 8.80             |      |                           |                                  |
| 29T         | U                  | 5.67             | 2.49 | 0.1780                    |                                  |
| 29NT        | U                  | 3.18             |      |                           |                                  |
| 30T         | M                  | 9.23             | 1.44 | 0.3680                    |                                  |
| 30NT        | U                  | 7.79             |      |                           |                                  |
| 31T         | U                  | 15.00            | 6.34 | 0.0123                    |                                  |
| 31NT        | U                  | 8.66             |      |                           |                                  |
| 32T         | U                  | 8.28             | 1.18 | 0.4429                    |                                  |
| 32NT        | U                  | 7.11             |      |                           |                                  |
| 33T         | U                  | 12.14            | 5.96 | 0.0161                    |                                  |
| 33NT        | U                  | 6.18             |      |                           |                                  |
| 34T         | U                  | 7.63             | 2.47 | 0.1811                    |                                  |
| 34NT        | U                  | 5.16             |      |                           |                                  |
| 35T         | M                  | 6.98             | 4.65 | 0.0398                    |                                  |
| 35NT        | M                  | 2.33             |      |                           |                                  |
| 36T         | M                  | 15.00            | 5.01 | 0.0310                    |                                  |
| 36NT        | M                  | 9.99             |      |                           |                                  |
| 37T         | U                  | 15.00            | 9.40 | 0.0015                    |                                  |
| 37NT        | U                  | 5.61             |      |                           |                                  |
| 38T         | M                  | 15.00            | 10.72| 0.0006                    |                                  |
| 38NT        | M                  | 4.28             |      |                           |                                  |
| 39T         | M                  | 7.90             | 1.10 | 0.4665                    |                                  |
| 39NT        | U                  | 6.80             |      |                           |                                  |
| 40T         | M                  | 15.00            | 7.48 | 0.0056                    |                                  |
| 40NT        | U                  | 7.52             |      |                           |                                  |
| 41T         | M                  | 8.97             | 1.22 | 0.4308                    |                                  |
| 41NT        | U                  | 7.75             |      |                           |                                  |
| 42T         | M                  | 9.25             | 2.17 | 0.2222                    |                                  |
| 42NT        | U                  | 7.08             |      |                           |                                  |
| 43T         | U                  | 15.00            | 8.77 | 0.0023                    |                                  |
| 43NT        | U                  | 6.23             |      |                           |                                  |
| 44T         | M                  | 8.76             | 0.22 | 0.8586                    |                                  |
| 44NT        | M                  | 8.54             |      |                           |                                  |
| 45T         | M                  | 8.92             | 2.86 | 0.1377                    |                                  |
| 45NT        | U                  | 6.06             |      |                           |                                  |
| 46T         | U                  | 10.34            | 2.17 | 0.2222                    |                                  |
| 46NT        | U                  | 8.17             |      |                           |                                  |
Table 2: Continued.

| Patient no. | SFRP3 methylation | $\Delta$Ct | $\Delta$Ct $\Delta$Ct tumor – $\Delta$Ct nontumor | SFRP3 tumor part Rel. to nontumor |
|-------------|-------------------|-------------|-------------------------------------------------|---------------------------------|
| 47T M       | 15.00             | 9.13        | 0.0018                                          |                                 |
| 47NT U      | 5.88              |             |                                                 |                                 |
| 48T U       | 15.00             | 9.16        | 0.0018                                          |                                 |
| 48NT U      | 5.85              |             |                                                 |                                 |
| 49T M       | 15.00             | 9.43        | 0.0014                                          |                                 |
| 49NT U      | 5.57              |             |                                                 |                                 |
| 50T M       | 15.00             | 6.78        | 0.0091                                          |                                 |
| 50NT U      | 8.22              |             |                                                 |                                 |
| 51T M       | 15.00             | 6.16        | 0.0140                                          |                                 |
| 51NT U      | 8.85              |             |                                                 |                                 |
| 52T M       | 15.00             | 10.77       | 0.0006                                          |                                 |
| 52NT U      | 4.24              |             |                                                 |                                 |
| 53T U       | 11.21             | 3.77        | 0.0733                                          |                                 |
| 53NT U      | 7.44              |             |                                                 |                                 |
| 54T M       | 12.27             | 10.28       | 0.0008                                          |                                 |
| 54NT U      | 1.99              |             |                                                 |                                 |
| 55T M       | 15.00             | 4.94        | 0.0326                                          |                                 |
| 55NT U      | 10.06             |             |                                                 |                                 |
| 56T U       | 7.74              | 1.98        | 0.2535                                          |                                 |
| 56NT U      | 5.76              |             |                                                 |                                 |
| 57T U       | 10.39             | 3.52        | 0.0872                                          |                                 |
| 57NT U      | 6.87              |             |                                                 |                                 |
| 58T M       | 15.00             | 9.01        | 0.0019                                          |                                 |
| 58NT U      | 5.99              |             |                                                 |                                 |
| 59T M       | 11.36             | 3.99        | 0.0632                                          |                                 |
| 59NT U      | 7.38              |             |                                                 |                                 |
| 60T M       | 7.52              | 2.55        | 0.1713                                          |                                 |
| 60NT U      | 4.97              |             |                                                 |                                 |

NT: nontumor part; T: tumor part; M: methylated; U: unmethylated.
The range given for SFRP3 tumor part relative to nontumor part is determined by evaluating the expression: $2^{-\Delta\Delta Ct}$.

Table 3: Frequency of SFRP3 promoter methylation in 30 control livers and 60 paired HCC and adjacent nontumor tissue samples.

| Diagnosis                    | No. of cases with SFRP3 methylation | $P$ value |
|------------------------------|-------------------------------------|-----------|
| Control livers* ($n = 30$)  | 0 (0%)                              |           |
| Chronic hepatitis ($n = 37$)| 6 (16.2%)                           | <0.0001   |
| Cirrhosis ($n = 23$)        | 9 (39.1%)                           |           |
| HCC ($n = 60$)              | 36 (60%)                            |           |

*Thirty control tissues were from 30 hepatic hemangiomas. Statistical analysis was determined by chi-square test.

3.2 Promoter Methylation of SFRP3 and Downregulation of SFRP3 mRNA in HCC Cell Lines. We then investigated the methylation level of SFRP3 promoter in four HCC cell lines (HA22T, HepG2, Hep3B, and TONG) using MSP and bisulfite sequencing. Among four HCC cell lines, our data demonstrated SFRP3 was fully methylated in HA22T cells and partially methylated in the other cells (Figure 2(a)). Bisulfite sequencing results were summarized in Figure 2(b). The CpGs in these regions was frequently methylated (Figure 2(b)). Quantitative RT-PCR data showed that down-regulation of SFRP3 mRNA in the four HCC lines with SFRP3 hypermethylation (Figure 2(c)). To confirm that the lack of expression of SFRP3 mRNA in the HCC lines was due to promoter hypermethylation, we treated cells with 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation. After treatment with 5 $\mu$M of 5-aza-2'-deoxycytidine, the unmethylated promoter DNA was detected by MSP and bisulfite sequencing; SFRP3 mRNA was restored or increased in the four HCC cell lines (Figures 2(a), 2(b), and 2(c)). These data indicate that hypermethylation of SFRP3 may be responsible for the absence or downregulation of mRNA transcription.
3.3. Downregulation of SFRP3 mRNA Is Correlated with Promoter Methylation in Primary HCCs. To study the relation between SFRP3 promoter methylation level and SFRP3 mRNA expression, we first checked the mRNA level of 60 primary HCCs and their corresponding adjacent nontumor tissues by quantitative RT-PCR. Our data showed SFRP3 mRNA expression was significantly downregulated in the primary HCCs as compared with the adjacent nontumor tissues ($P < 0.0001$) (Figure 3(a)). Next, we checked the methylation status of the HCC cell lines and clinical HCC tissues by QMSP. Hypermethylation was confirmed in the HCC tissues compared with the nontumor liver tissues ($P < 0.01$) (Figure 3(b)). In 36 of 60 HCCs (60%), SFRP3 mRNA was significantly downregulated (by >2-fold, Table 4). There was a statistically significant association between the down-regulation of SFRP3 mRNA and the methylation status of SFRP3 in HCCs (35/36 versus 17/24 resp.; $P < 0.01$) (Table 4). There were some HCCs without methylation; however, their SFRP3 mRNA expression were downregulated.

4. Discussion

Here we demonstrate that SFRP3 is significantly hypermethylated and downregulated in HCCs when compared with control livers and nontumor livers (containing chronic hepatitis or cirrhosis livers) ($P < 0.0001$, Table 3 and Table 2). SFRP3 mRNA expression could be restored or increased after HCC cells treatment with a DNA methyltransferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine (Figure 2). We found a significant correlation between methylation and transcription level in primary tissues (Table 4, $P < 0.001$).

In accordance with our data, promoter methylation has been detected in chronic hepatitis tissue and cirrhosis liver tissues, indicating that DNA methylation may be an early event in the pathogenesis of HCC [19, 40]. Put together, our data suggest that downregulation of SFRP3 mRNA through promoter hypermethylation is an early event during carcinogenesis and may be involved in the aberrant activation

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**Figure 1:** Methylation of SFRP3 in primary hepatocellular carcinoma tissues. (a) Schematic representation of the promoter region and the first exon of the SFRP3 gene. The CpG rich areas and the sites of methylation specific PCR (MSP), quantitative MSP, and bisulfitesequencing (BS) primers are indicated. (b) Representative results for four control livers (N1 to N5), four HCCs (T), and their corresponding nontumor livers (NT). Bisulfitel-modified genomic DNA was amplified using methylation-specific or unmethylation-specific primer sets. M, methylation-specific PCR product; U, unmethylation-specific PCR. DNA from the peripheral blood lymphocyte (PBL) sample was used as a negative control, and PBL DNA treated with SssI Methylase (New England Biolabs, Beverly, MA) was a positive control. Case numbers are indicated at the top. (c) Summary of bisulfitesequencing. Case numbers of tumors, nontumor tissues, and normal control are indicated at the top. Black and white circles correspond to methylated or unmethylated, respectively.

**Table 4:** Statistical correlation between SFRP3 mRNA expression and methylation status of SFRP3 CpG island in HCCs.

| Methylation of CpG island (no. of cases) | No methylation of CpG island (no. of cases) | $P$ value |
|-----------------------------------------|---------------------------------------------|-----------|
| Present                                 | 35                                          | 17        | $P < 0.01$ |
| Absent                                  | 1                                           | 7         |           |
of Wnt/β-catenin signaling in HCC. Moreover, SFRP3 mRNA was downregulated more than twofold in the absence of promoter hypermethylation in 71% of HCCs (17 of 24) (Table 4). The decreased SFRP3 mRNA level might be due to genetic changes or other epigenetic changes like histone modification.

Our data suggest that promoter hypermethylation of SFRP3 is a common event in HCCs and plays an important role in regulation of SFRP3 mRNA expression. Therefore epigenetic regulation of the Wnt/β-catenin pathway has been implicated as a possible therapeutic target in human cancer. Further investigations are required to explore the importance of SFRP3 in the development of hepatocellular carcinoma.

5. Conclusions

In conclusion, promoter hypermethylation of SFRP3 is a frequent event in HCCs and epigenetic downregulation of SFRP3 mRNA may contribute to aberrant activation of Wnt/β-catenin in HCC. This is the first report about hypermethylation and downregulation of SFRP3 mRNA in HCC.

**Abbreviation**

HCC: Hepatocellular carcinoma
SFRP3: Secreted frizzled-related protein 3
5-Aza-CdR: 5-Aza-2'-deoxycytidine
MSP: Methylation-specific PCR
RT-PCR: Reverse transcription-polymerase chain reaction
HBV: Hepatitis B virus
HCV: Hepatitis C virus
TSG: Tumor suppressor gene.
Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] F. X. Bosch, J. Ribes, R. Cléries, and M. Diaz, “Epidemiology of hepatocellular carcinoma,” *Clinics in Liver Disease*, vol. 9, no. 2, pp. 191–211, 2005.

[2] F. X. Bosch, J. Ribes, and J. Borràs, “Epidemiology of primary liver cancer,” *Seminars in Liver Disease*, vol. 19, no. 3, pp. 271–285, 1999.

[3] A. S. Befeler and A. M. Di Bisceglie, “Hepatocellular carcinoma: diagnosis and treatment,” *Gastroenterology*, vol. 122, no. 6, pp. 1609–1619, 2002.

[4] H. B. El-Serag, “Hepatocellular carcinoma: recent trends in the United States,” *Gastroenterology*, vol. 127, pp. S27–S34, 2004.

[5] H. B. El-Serag and A. C. Mason, “Rising incidence of hepatocellular carcinoma in the United States,” *The New England Journal of Medicine*, vol. 340, no. 10, pp. 745–750, 1999.

[6] M. D. Thompson and S. P. S. Monga, “WNT/β-catenin signaling in liver health and disease,” *Hepatology*, vol. 45, no. 5, pp. 1298–1305, 2007.

[7] T. Chiba, Y.-W. Zheng, K. Kita et al., “Enhanced self-renewal capability in hepatic stem/progenitor cells drives cancer initiation,” *Gastroenterology*, vol. 133, no. 3, pp. 937–950, 2007.

[8] G. Zeng, U. Apte, B. Cieply, S. Singh, and S. P. S. Monga, “siRNA-mediated β-catenin knockdown in human hepatoma cells results in decreased growth and survival,” *Neoplasia*, vol. 9, no. 11, pp. 951–959, 2007.

[9] J. Behari, “The Wnt/β-catenin signaling pathway in liver biology and disease,” *Expert Review of Gastroenterology and Hepatology*, vol. 4, no. 6, pp. 745–756, 2010.

[10] K. M. Cadigan and R. Nusse, “Wnt signaling: a common theme in animal development,” *Genes and Development*, vol. 11, no. 24, pp. 3286–3305, 1997.

[11] C. Y. Logan, J. R. Miller, M. J. Ferkowicz, and D. R. McClay, “Nuclear β-catenin is required to specify vegetal cell fates in the sea urchin embryo,” *Development*, vol. 126, no. 2, pp. 345–357, 1999.

[12] P. Polakis, “Wnt signaling and cancer,” *Genes and Development*, vol. 14, no. 15, pp. 1837–1851, 2000.

[13] C. Yost, M. Torres, J. R. Miller, E. Huang, D. Kimmelman, and R. T. Moon, “The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3,” *Genes and Development*, vol. 10, no. 12, pp. 1443–1454, 1996.
[14] J. Behrens, J. P. von Kries, M. Kühnl et al., “Functional interaction of β-catenin with the transcription factor LEF-1,” Nature, vol. 382, no. 6592, pp. 638–642, 1996.

[15] J. Cui, X. Zhou, Y. Liu, Z. Tang, and M. Romeih, “Wnt signaling in hepatocellular carcinoma: analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes,” Journal of Gastroenterology and Hepatology, vol. 18, no. 3, pp. 280–287, 2003.

[16] A. de La Coste, B. Romagnolo, P. Billuart et al., “Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas,” Proceedings of the National Academy of Sciences of the United States of America, vol. 95, no. 15, pp. 8847–8851, 1998.

[17] B. Yang, M. Guo, J. G. Herman, and D. P. Clark, “Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma,” American Journal of Pathology, vol. 163, no. 3, pp. 1101–1107, 2003.

[18] Y.-L. Shi, Y. Daigo, Y. Furukawa et al., “AXIN1 mutations in hepatocellular carcinoma, and growth suppression in cancer cells by virus-mediated transfer of AXIN1,” Nature Genetics, vol. 24, no. 3, pp. 245–250, 2000.

[19] H. Suzuki, E. Gabrielson, W. Chen et al., “A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer,” Nature Genetics, vol. 31, no. 2, pp. 141–149, 2002.

[20] J. G. Herman, A. Merlo, L. Mao et al., “Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers,” Cancer Research, vol. 55, no. 20, pp. 4525–4530, 1995.

[21] M. Esteller, A. Sparks, M. Toyota et al., “Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer,” Cancer Research, vol. 60, no. 16, pp. 4366–4371, 2000.

[22] J. Yu, H. Y. Zhang, Z. Z. Ma, W. Lu, Y. F. Wang, and J. D. Zhu, “Methylation profiling of twenty four genes and the concordant methylation behaviours of nineteen genes that may contribute to hepatocellular carcinogenesis,” Cell Research, vol. 13, no. 5, pp. 319–333, 2003.

[23] B. Yang, M. Guo, J. G. Herman, and D. P. Clark, “Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma,” American Journal of Pathology, vol. 163, no. 3, pp. 1101–1107, 2003.

[24] U. Schagdarsurengin, L. Wilkens, D. Steinemann et al., “Frequent epigenetic inactivation of the RASSF1A gene in hepatocellular carcinoma,” Oncogene, vol. 22, no. 12, pp. 1866–1871, 2003.

[25] H. Suzuki, E. Gabrielson, W. Chen et al., “A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer,” Nature Genetics, vol. 36, no. 4, pp. 417–422, 2004.

[26] P. W. Finch, X. He, M. J. Kelley et al., “Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action,” Proceedings of the National Academy of Sciences of the United States of America, vol. 94, pp. 6770–6775, 1997.

[27] E. J. Ekström, Y. Sherwood, and T. Andersson, “Methylation and loss of secreted frizzled-related protein 3 enhances melanoma cell migration and invasion,” PLoS ONE, vol. 6, no. 4, Article ID e18674, 2011.

[28] X. Zi, Y. Guo, A. R. Simoneau et al., “Expression of Frzb/secreted Frizzled-related protein 3, a secreted Wnt antagonist, in human androgen-independent prostate cancer PC-3 cells suppresses tumor growth and cellular invasiveness,” Cancer Research, vol. 65, no. 21, pp. 9762–9770, 2005.

[29] H. Hirata, Y. Hinoda, K. Ueno, S. Saini, and R. Dahiya, “Role of secreted frizzled-related protein 3 in human renal cell carcinoma,” Cancer Research, vol. 70, no. 5, pp. 1896–1905, 2010.

[30] Y.-L. Shi, C.-B. Hsieh et al., “SRFP1 suppressed hepatoma cells growth through Wnt canonical signaling pathway,” International Journal of Cancer, vol. 121, no. 5, pp. 1028–1035, 2007.

[31] J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin, and S. B. Baylin, “Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands,” Proceedings of the National Academy of Sciences of the United States of America, vol. 93, no. 18, pp. 9821–9826, 1996.

[32] S. Ogino, T. Kawasaki, M. Brahimandam et al., “Precision and performance characteristics of bisulfite conversion and real-time PCR (Methylight) for quantitative DNA methylation analysis,” Journal of Molecular Diagnostics, vol. 8, no. 2, pp. 209–217, 2006.

[33] H.-C. Lai, Y.-W. Lin, R.-L. Huang et al., “Quantitative DNA methylation analysis detects cervical intraepithelial neoplasms type 3 and worse,” Cancer, vol. 116, no. 18, pp. 4266–4274, 2010.

[34] W. B. Coleman and A. G. Rivenbark, “Quantitative DNA methylation analysis: the promise of high-throughput epigenomic diagnostic testing in human neoplastic disease,” Journal of Molecular Diagnostics, vol. 8, no. 2, pp. 152–156, 2006.

[35] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method,” Methods, vol. 25, no. 4, pp. 402–408, 2001.

[36] Y. Kondo, Y. Kanai, M. Sakamoto, M. Mizokami, R. Ueda, and S. Hirohashi, “Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation in 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma,” Hepatology, vol. 32, no. 5, pp. 970–979, 2000.