SPANDIDOS PUBLICATIONS

Methylation-associated silencing of SFRP1 in renal cell carcinoma

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Abstract. Secreted frizzled-related protein 1 (SFRP1) is a candidate tumor suppressor gene located at 8p11.2 and antagonizes the Wnt signaling pathway. Epigenetic inactivation of SFRP1 by methylation of its promoter CpG island has recently been reported in several types of cancers. In the present study, we examined the expression and methylation status of SFRP1 in renal cell carcinoma (RCC). Three RCC cell lines were tested and none expressed the SFRP1 transcript. Bisulfite sequencing of the SFRP1 promoter and treatment of the RCC cell lines with 5-aza-2'-deoxycytidine and/or trichostatin A revealed the association between SFRP1 expression and promoter hypermethylation. Methylation-specific PCR detected hypermethylation in 26/57 (45.6%) conventional RCC cases and 2/8 (25%) papillary RCC cases. Quantitative real-time PCR showed >3-fold decrease of SFRP1 expression in 33/34 (97.1%) conventional RCC cases. Microsatellite analysis showed loss of heterozygosity at the SFRP1 locus (D8S1180) in only 3/28 (10.7%) conventional RCC cases. The present findings indicate that methylation-associated silencing of SFRP1 frequently occurs in RCC and plays a pivotal role in early carcinogenesis. However, previous immunohistochemical studies on ß-catenin have suggested that activation of the canonical Wnt pathway through ß-catenin stabilization is infrequent in RCC. Thus, further basic studies are required to elucidate how the loss of SFRP1 activity contributes to the Wnt and other signaling pathways in RCC.

Introduction

Renal cell carcinoma (RCC) comprises of a heterogeneous group of tumors and is classified into various subtypes according to not only morphological features but also commonly observed genetic abnormalities (1). Conventional RCC is the major subtype of RCC and accounts for ~75% of kidney cancers: its development involves the accumulation of multiple chromosome deletion at 3p, 6q, 8p, 9p and 14q. Of these changes, loss of heterozygosity (LOH) at 3p is an early genetic change. The Von Hippel-Lindau gene is a tumor suppressor gene located at 3p.25 and its inactivation by mutation and hypermethylation has been reported in up to 57 and 19% of conventional RCCs, respectively (2). Additionally, LOH at 8p has been identified in 15-50% (3-5) and in particular, 8p21.1 deletions have been reported to be associated with tumor grade (5). Allelic loss of 8p is also a frequent genetic event in other types of cancers, suggesting the presence of one or more tumor suppressor genes on this chromosome arm (3).

Secreted frizzled-related protein 1 (SFRP1) is one of the candidate tumor suppressor genes located at 8p11.2 (6). SFRPs are extracellular signaling molecules that antagonize the Wnt signaling pathway: aberrations in this pathway are known to play a pivotal role in cancer development. Among members of the SFRP family, SFRP1 protein is expressed in the tubular cells of the normal kidney (7), which is the possible origin of RCC (1). A recent *in vitro* study showed that ectopic SFRP1 expression in SFRP1-deficient cancer cell lines attenuates Wnt signaling and induces apoptosis (8). In addition, epigenetic inactivation of SFRP1 by promoter hypermethylation has been reported in several types of cancers (6,9-11). Herein, we examined the expression and methylation status of SFRP1 in RCC and correlated the findings with clinical/pathological factors.

Materials and methods

Clinical samples. We obtained 65 tumor specimens and 22 corresponding normal renal tissues from 57 patients with conventional RCC and 8 patients with papillary RCC who had undergone surgery at Kyoto University Hospital. The tumors were staged and graded according to the International TNM classification system and the Fuhrman grading system, respectively (12,13). Each tissue specimen was snap-frozen immediately after surgical resection and was kept at -80˚C until DNA/RNA extraction. As controls for microsatellite analysis, peripheral blood leukocytes (PBLs) were collected from 33 patients with conventional RCC. Informed consent was obtained from all patients. The Institutional Review Board of the Kyoto University Graduate School of Medicine approved the present study.

Cell lines. Caki-1 and ACHN were purchased from American Type Culture Collection (Manassas, VA). NC65 was kindly donated by Dr F.H. Schroder (Erasmus University MC Rotterdam, The Netherlands) (14). These cell lines were derived from conventional RCC. All cell lines were maintained

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in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum.

RNA isolation and reverse transcription-PCR (RT-PCR). Total RNA was isolated using RNeasy kit and RNase-Free DNase (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA integrity was verified by using the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Reverse transcription was performed using 3 μg of total RNA and First-Strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). The primer sequences used were 5'-CTC AAC AAG AAC TGC CAC GC-3' and 5'-TTC ATC CTC AGT GCA AAC TCG-3' for SFRP1 and 5'-TAC CTG GTT GAT CCT GCC AGT-3' and 5'-ATC TAG AGT CAC CAA AGC CGC-3' for 18S rRNA.

Quantitative real-time PCR. For quantitative real-time PCR assay, QuantiTect SYBR-Green PCR kit (Qiagen) and GenAmp 5700 sequence detection system (Applied Biosystems, Foster, CA) were used according to the manufacturers' instructions. The PCR conditions were as follows: 95˚C for 15 min, followed by 40 cycles of 94˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. To verify that the primer pair used produced only a single band, a dissociation protocol was included after cycling and the dissociation of the PCR products was determined from 60˚C to 95˚C. The assay included a no-template control, a standard curve of 5 serial dilution points (in steps of 5- or 10-fold) of a control cDNA, and each of the test cDNAs. For each sample, the experiment was performed in duplicate and the mean Ct value was calculated. The gene expression level was calculated, as described previously (15). A cDNA sample pool of normal kidney tissue, derived from the specimens of 5 cases, was used as calibrator sample. The reference gene was that encoding TATA-binding protein (TBP). The primer sequences used were 5'-TCC CTG TGA CAA CGA GTT GAA A-3' and 5'-GGA CAA TCT TCT TGT CGC CAT T-3' for SFRP1 and 5'-CCC GAA ACG CCG AAT ATA AT-3' and 5'-CAC ACC ATT TTC CCA GAA CTG A-3' for TBP.

Demethylation and inhibition of histone deacetylation. The 3 RCC cell lines (ACHN, Caki-1 and NC65) were split to low density in 10-cm dishes and grown for 24 h. They were then treated with (i) 5-aza-2'-deoxycytidine (DAC; 10 μM; Sigma, St. Louis, MO) for 72 h, (ii) DAC (10 μM) for 72 h followed by Trichostatin A (TSA; 300 nM; Wako, Osaka, Japan) for an additional 24 h and (iii) TSA (300 nM) for 24 h. The medium containing DAC was changed every 24 h.

DNA extraction, bisulfite modification and sequencing. Genomic DNA was extracted as described previously (16) or by using QIAmp DNA mini kit (Qiagen). DNA was treated with sodium bisulfite by using Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol.

For bisulfite sequencing, the bisulfite-modified DNA was amplified by PCR with the following primers that were used in a previous study (9): 5'-TGG TTT TGT TTT TTA AGG GGT GTT GAG T-3' and 5'-TCC TAC CRC AAA CTT CCA AAA ACC TCC-3'. The primers span the 429 bp sequence around presumed transcriptional start site of SFRP1. The amplified products were then cloned into pCR2.1 vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The plasmid DNA was purified using QIAprep Spin mini prep kit (Qiagen) and then sequenced using Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Eight clones were sequenced for each sample.

Methylation-specific PCR (MSP). MSP was performed with the primers used in a previous study (9). The primer sequences were 5'-TGT AGT TTT CGG AGT TAG TGT CGC GC-3' and 5'-CCT ACG ATC GAA AAC GAC GCG AAC G-3' for the methylated reaction and 5'-GTT TTG TAG TTT TTG GAG TTA GTG TTG TGT-3' and 5'-CTC AAC CTA CAA TCA AAA ACA ACA CAA ACA-3' for the unmethylated reaction. The MSP conditions were as follows: denaturation at 95˚C for 5 min, 35 cycles of amplification (95˚C, 30 sec; 64˚C, 30 sec and 72˚C, 30 sec) and a final elongation step at 72˚C for 10 min. The PCR mixture contained 1X PCR buffer [67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$ and 10 mM ß-mercaptoethanol], 0.2 mM of deoxynucleotide triphosphate mixture, 0.5 μM of each primer, 1.0 units of JumpStart RedTaq polymerase (Sigma) and 1 μl of bisulfite-modified DNA in a final volume of 20 μl. The PCR products were analyzed by 3% agarose gel electrophoresis.

LOH analysis. The microsatellite marker D8S1180 was used with a fluorescence-labeled forward primer and a tailed reverse primer (Applied Biosystems). Genomic DNA extracted from normal kidney tissue or PBLs served as the normal control. The PCR products were electrophoresed on ABI 310 system and analyzed using GeneScan 3.1 software (Applied Biosystems). LOH was defined as >40% reduction in peak intensity compared to that observed for the corresponding normal control (17).

Statistical analysis. Fisher's exact test was performed to analyze the relationship between SFRP1 methylation status and clinical/pathological factors. The Mann-Whitney U test was used to analyze the correlation of SFRP1 expression with methylation status and clinical/pathological factors. Statistical significance was defined as $P<0.05$. Statistical analyses were performed using Dr SPSS II (SPSS, Chicago, IL).

Results

Re-expression of SFRP1 in RCC cell lines by demethylation and/or inhibition of histone deacetylation. None of the 3 RCC cell lines (ACHN, Caki-1 and NC65) expressed SFRP1 mRNA (Fig. 1). In ACHN, SFRP1 mRNA expression was induced after DAC or TSA treatment. In Caki-1, SFRP1 expression was restored after TSA treatment but not DAC treatment. In NC65, SFRP1 expression was detected after DAC treatment but not TSA treatment. In all the cell lines, the combination of DAC and TSA remarkably elevated the level of SFRP1 mRNA expression that had been induced by DAC or TSA.

Methylation status of SFRP1 in RCC cell lines. We analyzed the methylation status of the SFRP1 promoter in the 3 RCC

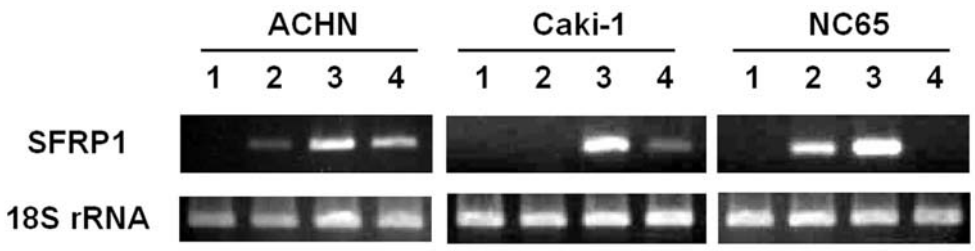

Figure 1. RT-PCR analysis of SFRP1 in RCC cell lines. The RCC cell lines were subjected to 4 different treatments: Mock (1), DAC (2), DAC + TSA (3) and TSA (4).

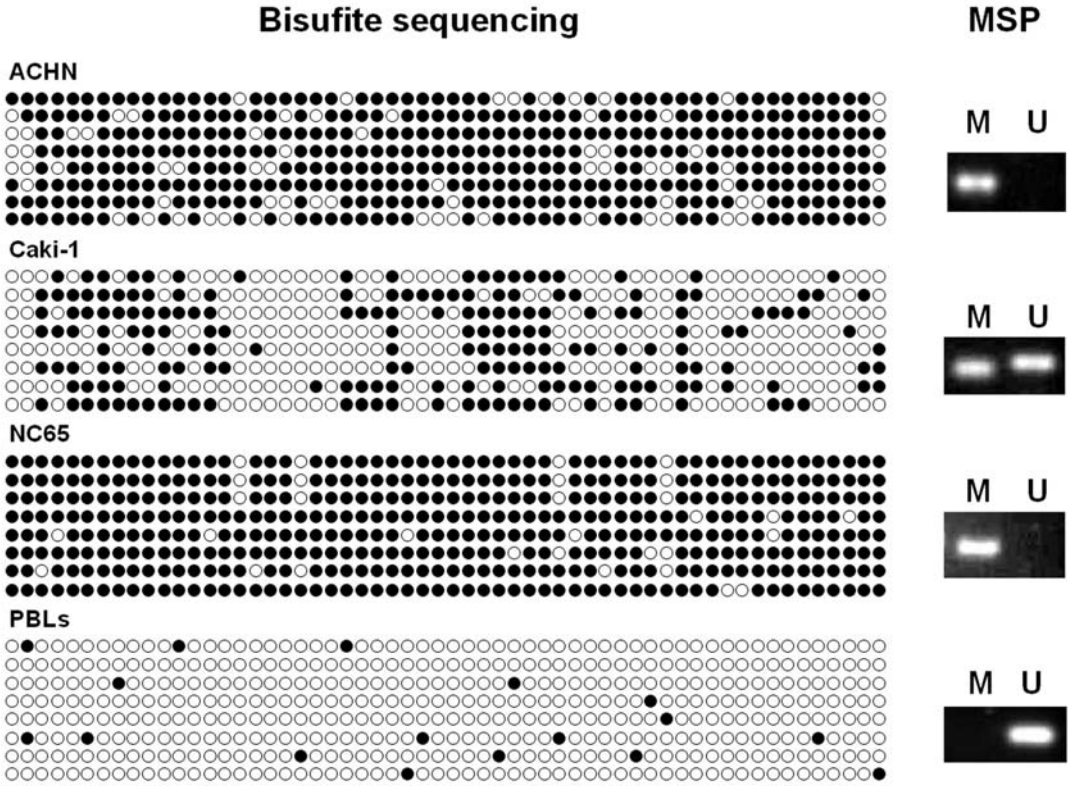

Figure 2. Bisulfite sequencing analysis of SFRP1 in the 3 RCC cell lines (ACHN, Caki-1 and NC65) and peripheral blood leukocytes (PBLs). Open and filled circles represent unmethylated and methylated CpG sites, respectively and each row represents a single clone. MSP results from the same samples are shown on the right. Bands in 'M' lanes are PCR products for methylation-specific primers and those in 'U' lanes are PCR products for unmethylation-specific primers.

cell lines by using bisulfite sequencing and MSP (Fig. 2). Bisulfite sequencing demonstrated that almost the entire promoter region was densely methylated in all the analyzed ACHN and NC65 clones. MSP detected methylated signals but not unmethylated signals in these cell lines. In Caki-1, bisulfite sequencing showed partial methylation of the promoter region and MSP detected both methylated and unmethylated signals. Then, we examined the methylation status of SFRP1 in PBLs from a healthy individual. Bisulfite sequencing and MSP showed virtually no methylation.

Methylation status of SFRP1 in primary RCC. By using MSP, we analyzed the methylation status of the SFRP1 promoter in the 65 primary RCCs and 22 normal kidney tissues (Fig. 3). NC65 and PBLs were used as the methylated and unmethylated controls, respectively. SFRP1 was methylated in 26 (45.6%) of 57 conventional RCC cases (Table I). Methylation status of SFRP1 was significantly associated with age (≥60 vs. <60: P=0.02), whereas it was not significantly related to gender, tumor stage and histological grade (gender: P=1.0, >T2 vs. T1 and T2: P=0.77, ≥N1 or M1 vs. N0M0: P=0.31, G1 and G2 vs. G3 and G4: P=0.16). In contrast, SFRP1 was methylated in 2 (25%) of 8 papillary RCC cases.

SFRP1 expression in primary RCC. We analyzed the SFRP1 transcriptional levels in 34 clinical samples of conventional RCC by using quantitative real-time PCR. In 33 (97.1%) of 34 conventional RCC samples, SFRP1 expression was down-regulated by >3-fold compared to that in the normal kidney tissue (Table I). The expression levels of SFRP1 were not significantly associated with SFRP1 methylation (P=0.41) and clinical/pathological factors (≥60 vs. <60: P=0.54, gender: P=0.63, >T2 vs. T1 and T2: P=0.90, ≥N1 or M1 vs. N0M0: P=0.11, G1 and G2 vs. G3 and G4: P=1.0).

Table I. Summary of clinical/pathological factors and SFRP1 status in RCC patients.

No.	Age	Gender	Stage	Grade	Methylation		LOH	Expression (vs. normal)
					Cancer	Normal		
Conventional RCC								
1	62	M	T2N0M0	4	M		NI	5.26E-04
2	72	M	T1bN0M0	2	M	U	-	7.02E-04
3	61	F	T1bN0M0	2	U	U	NI	1.14E-03
4	71	M	T1bN0M0	3	M	U	NI	1.15E-03
5	71	F	T3bN0M0	4	M		-	1.22E-03
6	54	M	T1bN0M0	2	U		-	1.48E-03
7	39	M	T3aN0M0	2	U	U	-	1.74E-03
8	76	F	T3bN0M1	2	M	U	-	2.25E-03
9	66	M	T1aN0M0	2	M		-	2.40E-03
10	67	M	T1aN0M0	2	M		NI	2.95E-03
11	64	M	T1bN0M0	2	M	U	-	3.30E-03
12	44	M	T2N0M0	2	U	U	NI	3.35E-03
13	74	M	T1aN0M0	1	U	U	NI	3.52E-03
14	60	M	T1aN0M0	2	U		NI	3.78E-03
15	65	M	T1aN0M0	2	U		NI	4.53E-03
16	64	M	T3aN0M1	3	U		-	4.75E-03
17	60	M	T1aN0M0	2	U	U	-	4.85E-03
18	55	M	T1bN0M0	2	M		-	4.99E-03
19	74	M	T1aN0M0	2	M		-	5.64E-03
20	67	F	T1bN0M0	2	M	M	+	6.97E-03
21	67	M	T1aN0M0	1	U	U	-	1.03E-02
22	46	M	T1aN0M0	2	U		NI	1.19E-02
23	58	F	T2N0M1	2	U		NI	1.21E-02
24	49	F	T1bN0M0	2	U		NI	1.27E-02
25	60	F	T3bN0M0	2	U		-	1.70E-02
26	63	M	T3bN0M1	3	M		-	3.55E-02
27	69	M	T3aN0M1	2	U		+	4.33E-02
28	38	F	T1bN0M0	3	U	U	NI	8.62E-02
29	66	M	T3bN1N1	4	M	U	-	8.99E-02
30	70	M	T2N0M0	1	M	U	NI	9.04E-02
31	60	M	T2N2M1	4	M		-	1.12E-01
32	74	M	T1aN0M0	2	U		NI	2.15E-01
33	65	M	T1aN0M0	2	M	U	NI	2.96E-01
34	67	F	T1aN0M0	2	U		NI	1.18E+00
35	73	F	T1bN0M0	2	M	M	NI	
36	72	M	T1aN0M0	1	U	U	-	
37	63	M	T1aN0M0	2	U	U	-	
38	37	M	T2N0M0	1	M	U	-	
39	76	F	T1aN0M0	1	U	U	-	
40	69	M	T3aN0M0	2	U	U	NI	
41	67	F	T3aN0M0	2	U	U	-	
42	61	M	T3aN0M0	2	U		N	
43	69	M	T3aN0M1	2	M		NI	
44	61	M	T1bN0M0	3	U		NI	
45	64	F	T2N0M0	2	M		+	
46	64	M	T1aN0M0	2	M		ND	
47	74	F	T2N0M0	3	M		NI	
48	32	M	T2N0M0	1	U		-	
49	84	F	T1aN0M0	2	M		NI	
50	60	M	T3bN0M0	2	M		-	

Continued.

No.	Age	Gender	Stage	Grade	Methylation		LOH	Expression (vs. normal)
					Cancer	Normal		
Conventional RCC								
51	64	M	T3bN0M0	2	U		-	
52	47	M	T3aN0M0	2	U		NI	
53	55	F	T1aN0M0	2	U		ND	
54	68	M	T1bN0M0	2	U		-	
55	70	M	T3aN0M1	2	M		-	
56	59	M	T3bN0M1	2	U		NI	
57	65	M	T2N0M1	2	M		NI	
Papillary RCC								
1	72	M	T1bN0M0	2	U			
2	53	M	T1aN0M0	2	U			
3	67	F	T1bN0M0	3	M			
4	75	M	T3aN1M1	2	U			
5	57	M	T1bN0M0	2	U			
6	79	M	T1aN0M0	2	U			
7	74	M	T1aN0M0	2	M			
8	76	M	T1aN0M0	1	U			

M, methylated; U, unmethylated; NI, not informative and ND, not determined.

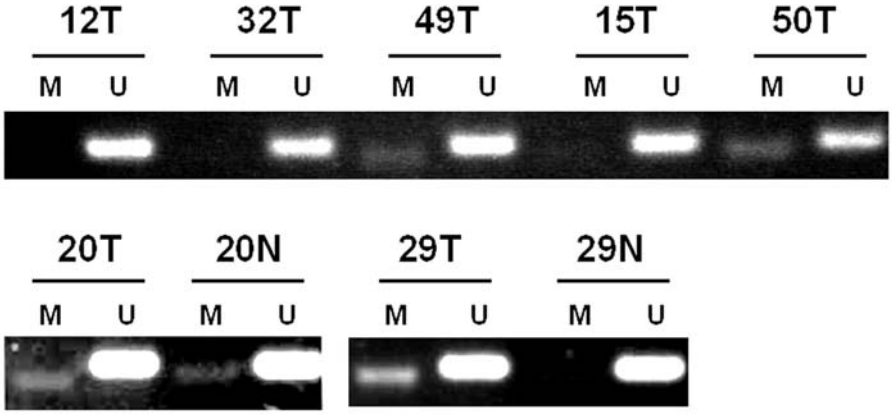

Figure 3. MSP analysis of SFRP1 in primary conventional RCC. The number corresponds to that in Table I. T and N denote tumor and normal kidney, respectively. Bands in the 'M' and 'U' lanes are as indicated in Fig. 2.

Analysis of LOH at D8S1180. To determine whether primary RCC shows allelic loss of the SFRP1 locus, we performed LOH analysis by using the microsatellite marker D8S1180, which is located only 6 kbp telomeric to the SFRP1 gene. Among 28 informative cases of the 55 conventional RCC specimens, only 3 (10.7%) samples showed allelic loss of D8S1180 (Table I).

Discussion

Hypermethylation in the CpG island of the SFRP1 promoter has recently been reported in several types of cancers including those of colon (6,9), lungs (10) and ovaries (11). Here, we detected SFRP1 methylation in 26 (45.6%) of the 57 conventional RCC samples. While our study was in preparation, three groups reported frequent SFRP1 methylation (46.8-80%) in clinical RCC samples (18-20), which is consistent with the current result. In the present study, SFRP1 was remarkably down-regulated in most of the conventional RCC samples, irrespective of the SFRP1 methylation status. Additionally, Dahl *et al* showed the frequent reduction of SFRP1 expression in the SFRP1 unmethylated samples although SFRP1 methylation status was significantly associated with mRNA expression in their study (P=0.048) (19). There could be several reasons underlying these findings. The samples were split for DNA and RNA extractions. Thus,

heterogeneity in the samples may cause these findings. Then the samples contained both cancer cells and other cell types, which may have hindered the detection of SFRP1 methylation. Furthermore, not only promoter hypermethylation but also histone modification plays a critical role in epigenetic silencing. Zhao *et al* demonstrated that O^6-methylguanine-DNA methyltransferase, essential for DNA repair, is epigenetically silenced without hypermethylation in several cancer cell lines (21). In the present study, Caki-1, an SFRP1 non-expressed cell line, re-expressed SFRP1 mRNA not by DAC treatment but by TSA treatment and the CpG island of SFRP1 promoter in Caki-1 was not densely methylated. Thus, in some cases of conventional RCC, SFRP1 may be epigenetically silenced by histone modification without promoter hypermethylation.

The present study showed that the methylation status of SFRP1 was significantly associated with age. Although this finding needs to be confirmed in further studies, aging may have some influence on the process of SFRP1 epigenetic inactivation in RCC.

Previous studies demonstrated SFRP1 methylation in some of the normal tissues from colon carcinomas (6,9). We also detected SFRP1 methylation in 2 out of 22 normal kidneys from RCC patients. Given that SFRP1 expression was remarkably reduced in the majority of the RCC specimens, this finding suggests that the epigenetic silencing of SFRP1 is an early event in RCC carcinogenesis.

LOH at 8p12-22 has been detected in 15-50% of conventional RCC cases (3-5). In the present study, allelic loss of the SFRP1 locus (8p11.2) was infrequently observed (10.7%). Gene deletion may not be important in the reduction of SFRP1 expression. Furthermore, we detected SFRP1 methylation in 2 out of 8 papillary RCC cases. Recently, Yamazaki *et al* identified SFRP1 as the gene that is commonly down-regulated in conventional, papillary and chromophobe RCCs by using cDNA microarray (22). Thus, the epigenetic inactivation of SFRP1 might be a common phenomenon in the 3 types of RCC.

Signals of the Wnt pathway are transduced by Wnt ligands through frizzled (Fz) membrane receptors. SFRPs attenuate these signals by competing with Fzs for Wnt ligands or by directly associating with Fzs. On binding to the Fz receptors, Wnt ligands can activate the canonical or non-canonical Wnt pathway (6). The canonical Wnt pathway operates via stabilization and increased transcriptional activity of ß-catenin. However, immunohistochemical studies on ß-catenin have shown that activation of the Wnt signal through ß-catenin is not a significant event in RCC (23,24). On the other hand, the non-canonical Wnt pathway can activate the c-Jun N-terminal kinase. Oya *et al* suggested that c-jun activation is relevant to early RCC carcinogenesis (25). In addition, the Wnt signal has been reported to activate the extracellular signal regulated-kinase (ERK) pathway in either a ß-catenin-dependent or -independent manner (26). Previously, our group showed that ERKs are frequently activated in RCC (27). Thus, the down-regulation of SFRP1 in RCC may contribute to the activation of the non-canonical Wnt and ERK pathways rather than the canonical Wnt pathway. Further basic studies are required to evaluate the contribution of SFRP1 inactivation to the Wnt and ERK pathways in RCC.

Acknowledgements

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