The Wnt Antagonist sFRP1 in Colorectal Tumorigenesis

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ABSTRACT

Regions of the short arm of chromosome 8p occur in a range of solid tumors, indicating that tumor suppressor genes reside at these loci. In this study, we have examined the properties of the Wnt signaling antagonist secreted frizzled-related protein (sFRP) 1 as a candidate for this role at c8p11.2. An initial survey of 10 colorectal tumors, selected by the presence of isolated short deletions of the 8p11.2 region, identified three chain-terminating mutations, all within the first exon, which encodes the cysteine-rich domain. None of these tumors exhibited microsatellite instability, indicating intact mismatch repair gene function. The preserved sFRP1 alleles in the remaining seven tumors each contained a polymorphic three-base insertion in the signal sequence, but in a broader study, no association was found between this and the development of colorectal cancer. Epigenetic inhibition of sFRP1 transcription was investigated, and increased methylation of the promoter region was demonstrated in an additional cohort of 51 locally advanced colorectal cancers. Methylation was identified in 50 (98%) of the cancers and in only 11 of 36 (30%) matched normal mucosal samples (P < 0.001). Semi-quantitative analysis, by real-time PCR, of mRNA expression in 37 of the same cohort of 51 cancers revealed that sFRP1 mRNA expression was down-regulated in 28 (76%) cases compared with matched normal large bowel mucosa. The 3’ end of the sFRP1 mRNA also was found to be alternatively spliced, compared with the prototype liver and lung forms, in the colon and a number of other tissues, yielding an extended COOH terminus, which may influence its activity in a tissue-specific manner. The inactivation and down-regulation of sFRP1 observed are consistent with it acting as a tumor suppressor gene in colorectal carcinogenesis. Because β-catenin is constitutively active in the majority of colorectal tumors, it is unlikely that sFRP1 can act in the canonical Wnt response pathway. Therefore, we propose that the reduced activity or absence of sFRP1 allows the transduction of noncanonical Wnt signals, which contribute to tumor progression.

INTRODUCTION

Interstitial deletions of chromosome 8p occur at a high frequency in a range of different cancers (1–7), and linkage studies indicate the breast cancer gene BRCA3 may reside on this arm (8, 9). Our studies of the region 8p11.2 in a series of prostate cancers (10), squamous cell head and neck cancers (11), and colorectal carcinomas (12) have shown frequent interstitial deletions in these different tumor types and an association between 8p11.2 deletion and local invasion (10, 12). We also have identified a minimum region of deletion between the gene and the STS marker D8S515. The localization of sFRP1 to 8p11.2 and its function as an antagonist of Wnt signaling led us to investigate this gene as a candidate tumor suppressor gene (13).

The first mammalian Wnt was identified by its ability to promote mouse mammary tumorigenesis (14). The canonical Wnt response pathway operates by stabilizing β-catenin, enabling it to accumulate in the nucleus where it directs transcription of a range of genes in association with Lef/TCF factors (15). In colorectal cancer, inactivating mutations of APC or stabilizing mutations of β-catenin lead to constitutive activation of this pathway, and this has led to the assumption that Wnt signaling can make no additional contribution to tumor progression (16).

However, two β-catenin-independent Wnt response pathways have been described more recently. The planar cell polarity pathway, first identified in Drosophila (17), diverges from the β-catenin response upstream of APC and leads to activation of RhoA and JNK, whereas the Wnt/Ca pathway operates through PKC and CamKII (18). It is possible that at least one of these contributes to tumor progression because Wnt3 has been shown to direct cyclooxygenase 2 transcription through a β-catenin-independent route (19).

β-catenin regulates the expression of a number of Wnt pathway factors, including Axin 2 and hNkd1 (20), the latter acting to direct the response toward the planar cell polarity pathway (21). Thus, dysregulation of β-catenin activity is likely to lead to a redirection of subsequent Wnt responses toward the noncanonical pathways, and this may play a role in progression of the tumor.

Wnt inhibitory factor 1 (22), Dickkopfs (23), and the secreted frizzled-related proteins (sFRPs; Refs. 13, 24) are distinct classes of extracellular Wnt antagonists and may be expected to counter persistent or excessive stimulation by Wnts. Dickkopfs genes do not interact directly with Wnts but block the action of the lrp 5/6 coreceptor in canonical signaling (25), whereas Wnt inhibitory factor 1 may be specific for Wnt8 (22).

The sFRPs comprise an N-terminal domain homologous to the cysteine-rich domain (CRD) of the frizzled family of Wnt receptors and a COOH-terminal domain with some homology to netrin (13). The CRD domain of the sFRPs competes with the frizzled receptors for Wnt binding, modulating the signal (26).

The sFRP1 gene was proposed to lie at 8p11.2, within the region found to be deleted in our earlier tumor studies. Loss of expression has been shown recently to correlate with lymph node metastases and increased mortality in breast tumors (27).

We present data on the localization, gene structure, a frequent polymorphism in the NH2 terminus of sFRP1, and tissue-specific alternative splicing of the COOH terminus. Mutational analysis of the coding region demonstrated homozygous inactivating mutations in 30% of a selected series of colorectal tumors, indicating that sFRP1 might act as a tumor suppressor gene in colorectal carcinogenesis. We proceeded to investigate sFRP1 in a larger cohort of colorectal tumors, looking at the influence of epigenetic phenomena on expression levels, and genomic alterations.

MATERIALS AND METHODS

Microsatellite Instability Analysis. Microsatellite instability was studied at five loci: BAT26 and BAT40, which are mononucleotide repeat markers, and D2S123, D8S255, and D13S175, which are dinucleotide repeat markers. Oligonucleotides used were as follows: BAT26 forward, 5′-TGACTACTTTGACT-TCAGCC; BAT26 reverse, 5′-AACCATTCAACATTTTAAACC; BAT40 for-

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ward, 5′-ATTAATCTTCATACCCACC; BAT40 reverse, 5′-GTAGAGC- AAGGACCCCTTTG-3′; D2S123 forward, 5′-ACATTTGCTGAGGTTGCTG; D2S123 reverse, 5′-CTTCTCTGACTGTTGATCCA; D8S255 forward, 5′-TTTT- TGTAGTTCTGTTCC; D8S255 reverse, 5′-TGAAACCCACAGATATT- GGG; D13S175 forward, 5′-TATTTGGACCTTGGATTTGTCG and D13S175 reverse, 5′-TGATCGTACCCACATAGGTTA. Bat26 and Bat40 were labeled fluorescently with tetrachloro-6-carboxyfluorescein, and D8S255 was labeled with hexachloro-6-carboxyfluorescein.

PCR conditions for all of the reactions were as follows: 95°C for 5 min, then 24 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products (1 µl) were combined with 1.5 µl loading buffer, size standard 0.5 µl 6-carboxytetramethylrhodamine, and 1 µl formamide (all supplied by Applied Biosystems, Foster City, CA). Products were denatured at 95°C for 4 min and resolved on an ABI 377 6% denaturing polyacrylamide gel (19:1; Applied Biosystems). Results were analyzed using ABI Genescan software (Applied Biosystems).

Immunohistochemistry. The streptavidin-biotin indirect immunoperoxidase method was used as we have published previously (28). Briefly, 5-µm sections were dewaxed, rehydrated, and blocked by 10% H2O2 in methanol for 10 min. Microwave antigen retrieval was undertaken for 1 h. Sections were incubated overnight with primary antibody. Primary antibodies hMLH1 and hMSH-2 were used at a dilution of 1:20 and 1:100, respectively (PharMingen, San Diego, CA). After washing with PBS, sections were incubated with biotinylated goat antimouse (Dako, Carpinteria, CA) according to the manufacturer’s instructions for 30 min. Serial PBS washing and incubation with streptavidin-peroxidase conjugate (Dako) was undertaken before incubation with diaminobenzidine tetrahydrochloride (Sigma, Poole, United Kingdom). Sections were counterstained with hemalum, dehydrated, and then analyzed by light microscopy.

Loss of Heterozygosity Analysis. The colorectal tumors were examined for loss of heterozygosity using four microsatellite markers (D5S305 forward, 5′-AGGCTGCCATTTGTGATAAGTT; reverse, 5′-AGTGCCTGGCTAAGAG; D8S532 forward, 5′-GCTCAAGGCTCTCAATGAG; reverse, 5′-GACCCTGTGACACCCTG; D8S519 forward, 5′-CTGTCCACCCACGGCT; and reverse, 5′-AGGAGGCTTTCGGTTCC) found on chromosome 8p11.2 close to the sFRP1 gene. Oligonucleotide primers were obtained from Alta Biosciences (University of Birmingham, Birmingham, United Kingdom).

All of the PCR reactions were prepared to a final volume of 25 µl, containing 2.5 µl of 10× BIOTAQ NH4-based reaction buffer (Bioline, Randolph, MA), 1 mm MgCl2, 0.2 mm deoxynucleotides triphosphate stock (Amersham Pharmacia Biotech, Piscataway, NJ), 5 pmol forward primer, 5 pmol of unlabeled reverse primer, 0.5 units of BIOTAQ DNA polymerase (Bioline), and 100 ng of genomic DNA. Amplification involved an initial step of 2 min at 94°C, followed by 35 cycles of 94°C for 10 s, 55°C or 59°C for 20 s, and 72°C for 30 s, with a final extension of 5 min at 72°C. The DNA was denatured and separated on a 6% acrylamide gel followed by autoradiography.

Analysis of Promoter Methylation Status. The method used for DNA modification was essentially that of Grunau et al. (29). In short, 10 µg RNA (Sigma) were added to 1 µg of genomic DNA and made up to 100 µl. Freshly prepared NaOH (Sigma) was added to a final concentration of 0.3 M, and the sample was incubated at 42°C for 20 min. A total of 1.2 ml 5.2–5.69 M sodium bisulfite (Sigma) and 10 mM hydroquinone (Sigma; pH 5) was added, and the solution was overlaid with mineral oil and incubated at 55°C for 4 h. DNA was desalted and redissolved into 100 µl of Tris-Cl (pH 8; Sigma). NaOH was added to a final concentration of 0.3 M, and the solution was then incubated at 37°C for 20 min. After incubation, the solution was neutralized; 10 µg of RNA were added; and nucleic acids were precipitated with ethanol at −20°C overnight. Precipitated DNA was washed with 70% ethanol, dried, and resuspended in 50 µl of 1 M Tris-HCl (pH 8).

Modified DNA was amplified with oligonucleotides specific for unmethylated DNA (forward, 5′-GAGTGATTGTGTGTGTGTGTGTGTGTGT; reverse, 5′-CCCACATCCACCACCTCCACACTG) with cycle conditions of 95°C for 5 min, 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min, and 72°C for 7 min. Oligonucleotides specific for methylated DNA were used forward 5′-GTTGCGGCGGCTGCGCCTGCTCC and reverse 5′-AAGGTTACCCGACTCCGGAGCC. Cycle conditions were the same as with unmethylated DNA except for an annealing temperature of 63°C. Amplified products were analyzed on a 2% agarose gel.

Methylation status also was analyzed using the combined bisulfite restriction analysis (COBRA) method (30). The sFRP1 CpG island region was predicted using CpG plot (www.ebi.ac.uk). A strong CpG island (island size > 100 bp; GC percent > 50%; and Obs/Exp > 0.6) was detected within the region −180 bp to +530 bp relative to the transcription start site of the sFRP1 gene. To analyze this region of the gene for methylation, PCR primers were designed that were specific for the bisulfite modified sequence. The sFRP1 CpG island was analyzed using nested PCR with the following primers: sFRP1 COBRA forward, 5′-GGTTAGTGTGGTGGTCTGTGTTA and sFRP1 COBRA reverse, 5′-CCTTACCTTTAAAAAATCAGTCT. The PCR conditions used in both reactions were 94°C for 3 min; 30 cycles of 94°C for 20 s, 54°C for 30 s, 72°C for 40 s, and 72°C for 7 min; and 35 cycles were used in the nested PCR reaction. PCR products were incubated with restriction enzymes TaqI and BstUI for 2 h at 65°C and 60°C, respectively, to assay for methylation and were visualized on a 2% agarose gel.

Semiquantitative Real-Time PCR. Total RNA was extracted using TRI reagent (Helena Biosciences, Sunderland, United Kingdom). First-strand cDNA was synthesized from 2 µg of DNase-treated total RNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech) and random hexamers (Promega, Madison, WI).

Oligonucleotide primers and TaqMan probes were designed using Primer Express, version 1.5 (Applied Biosystems). The primers for sFRP1 (NM_003012) gene amplification were 5′-CCATCCACCCAGGAAGCT and 5′-CTGAGCTCTCATCAGTCCACCTC. The sequence for the TaqMan fluorescent probe for sFRP1 was 5′-CAAGGCCCCAACGACACACGTTG. Data for the sfrp1 gene were normalized to the epithelial cell-specific gene keratin 8 (KRT8; NM_002273). For KRT8, the primers and probe were 5′-GATGCGC- CACTAACGAGACGCT, 5′-ACTATGTTCTGTCATGCCAGACT, and 5′- CCGGCGTTCTCCGTCCCTCCA, respectively. The TaqMan Universal PCR Master Mix and the TaqMan probes were purchased from Applied Biosystems. Primers were obtained from Alta Biosciences (University of Birmingham). Multiplex PCR amplifications were performed using an ABI PRISM 7700 sequence detector in a final volume of 25 µl (Applied Biosystems). Each reaction contained 12.5 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 90 nm KRT8 and sFRP1 primers, 150 nm sFRP1 TaqMan probe, 175 nm KRT8 TaqMan probe, 1 µl of cDNA sample, and water. The thermal cycling conditions comprised an initial step at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Informed consent was obtained from each patient for molecular analysis of the resected specimen. To analyze differences between the tumor and normal specimens, χ2 test was used.

RESULTS

The Genomic Map of sFRP1. A pool of PAC clones, already localized to 8p11.2, was screened for sFRP1. This identified two clones, 100H11 and 152M3, which contained the entire coding sequence of sFRP1, as judged by their ability to support amplification of 5′- and 3′-specific amplicons. Fluorescence in situ hybridization analysis of selected colorectal tumors, using these PAC clones as probes, confirmed them to lie within the deletion region at 8p11.2. These clones contained the STS marker D8S268, and deletion was confirmed in 30% of squamous cell head and neck carcinomas (data not shown).

The PAC clones were used to confirm the genomic map of sFRP1. A combination of PCR amplification and cycle sequencing revealed the presence of two introns in the coding sequence at nucleotides 540/541 and 618/619, as shown in Fig. 1.

This map corresponds to the expected domain structure of the sFRP1 protein. The first coding exon contains the whole of the frizzled-related CRD, whereas the third exon contains the netrin-related domain (31). The short middle exon probably represents a spacer between these two.
Identification of Protein-Truncating Mutations in Tumor DNA.

Flanking primers were designed to amplify each exon from genomic DNA for analysis by direct cycle sequencing. Genomic DNA was analyzed from 10 advanced colorectal tumors shown previously to carry heterozygous interstitial deletions at 8p11.2. Mutations leading to premature termination of the translation product were identified in 3 of the 10 samples. These were two single-base deletions (26delG and 67delG) and a single-base change (G450A), generating an in-frame stop codon (Fig. 2B). Each of these mutations was found within the first exon, shown previously to be sufficient for Wnt antagonist activity (32, 26). Of the 10 tumors analyzed, none contained truncating mutations in the second or third exons of sFRP1.

The possibility that the mutations had been generated by a mutator genotype was investigated. MMR protein expression (hMLH-1 and hMSH-2) was investigated in each tumor by immunohistochemistry. The presence of microsatellite instability was investigated using five different mono/dinucleotide repeat markers (BAT26, BAT40, D2S123, D8S255, and D13S175). No loss of protein expression was detected, and no instability at a DNA level was observed in any of these 10 tumors, indicating the mutations were unlikely to have arisen as a consequence of failure of MMR gene function.

An additional 51 tumors were analyzed by direct sequence analysis, of which 49 provided clearly interpretable results. Only the first exon was sequenced for stop codon mutations, but none were found. The remaining exons were not sequenced because no mutations had been identified in those regions in the preliminary analysis. This finding indicated that point mutation is not a frequent method of inactivation of the sFRP1 gene in colorectal cancer. The same bank of 51 locally advanced (T3/4) tumors then was analyzed for evidence of other methods of DNA disruption.

Increased Methylation of the sFRP1 Promotor Region in Tumor DNA.

The possibility that sFRP1 expression was modified by epigenetic factors was investigated in the same cohort of 51 cancers (Table 1), of which 49 gave interpretable results. Investigation of the
methylation status of the promoter region of sFRP1 by methylation-specific PCR and COBRA (shown in Fig. 3) revealed hypermethylation in 40 (82%) cancers. Although these two approaches agreed broadly, COBRA was more sensitive and informative. We proceeded to analyze a selection of the matched normal colonic mucosal samples (n = 36; Table 1). Gels were scored by eye for the presence or absence of a methylated band, and images were quantified using the GeneTools analysis package (Syngene, Cambridge, United Kingdom) to provide the percentages given in Table 1. There were no cases in which sFRP1 was unmethylated in the tumor but methylated in the matched normal mucosa. Only 11 methylated matched normal mucosa samples (P < 0.001) were found. The mean (median) level of DNA modification differed between the groups, at 35% (33%) for the cancers and 10% (9%) for normal mucosa.

These data demonstrate that hypermethylation of the sFRP1 promoter region is a frequent event in colorectal cancer and is increased significantly compared with normal mucosa from the same patient.

The 3' End of the sFRP1 Coding Region Is Alternatively Spliced. Attempts to amplify sFRP1 from colonic mucosa cDNA with 3' primers immediately downstream of the stop codon failed, whereas more distal primers yielded amplicons smaller than the predicted size (Fig. 4). Sequencing revealed that nucleotides 913-1005 were absent from this transcript, removing seven amino acids and the stop codon predicted in the original sequence and extending the reading frame by an additional 40 amino acids, terminating at nucleotide 1125.

A review of the splicing patterns in cDNAs from a range of tissues (Fig. 4) revealed that the extended protein is the predominant species. The unspliced form is the major species in only the lung and liver, whereas the prostate expresses another variant, which maintains the stop codon used in liver but lacks sequences from nucleotides 942-1092, downstream of the stop codon. Heart cDNA contains the colon and the prostate forms at low, but approximately equivalent, levels.

The extended sequence contains a hydrophobic region, which may act as a transmembrane anchor, modifying the localization of the protein. This may influence the function of sFRP1 in different tissues because an unspliced protein may be more effective in antagonizing Wnt signaling to tumor cells in trans than would a membrane-bound form.

sFRP1 Transcription Is Down-Regulated in Colorectal Tumors. We compared the sFRP1 transcript expression level in the same cohort of tumors. Thirty-seven colorectal tumor samples with matched normal mucosa gave analyzable RNA (Table 1; Fig. 5). The sFRP1 TaqMan probe was designed to span the exon1/intron1 boundary of the gene. sFRP1 expression in each tumor and normal colon sample was standardized to cytokeratin 8 (KRT8) gene expression. KRT8 was used as an epithelial cell-specific marker because stromal and inflammatory cell components may vary in tumor and matched normal epithelium. The expression of KRT8 was consistent between normal and tumor samples. Expression of sFRP1 in tumors was normalized to the mean of sFRP1 expression in the matched normal mucosa.

As shown in Fig. 5, sFRP1 mRNA was down-regulated by >10-fold in 28 of the 37 (76%) tumors compared with normal mucosa. In 6 of 37 (16%) tumors, there was a <10-fold change in expression level, and sFRP1 expression was up-regulated in 3 of 37 (7%) tumors.

The clinical and pathologic data from these 37 tumors were investigated, but no correlation was found between sFRP1 expression level and patient age, sex, tumor site, serosal spread, or presence of lymph node metastases.

**DISCUSSION**

Our studies indicate that sFRP1 acts as a tumor suppressor gene in colorectal carcinogenesis, as demonstrated by homozygous inactivation through interstitial deletion and truncating mutations. We also have identified that hypermethylation of the promoter region is a frequent event in a series of 51 locally advanced colorectal cancers. Reduced transcript levels were seen in >75% of these cases.

Our previous studies (10–12) showed localized deletion of the
sFRP1 locus in advanced colorectal, prostate, and squamous head and neck tumors, indicating that it plays a role in the progression of many solid tumors. Other studies have demonstrated reduced transcription of sFRP1 in breast cancer (27, 33, 34) and gastric cancer (35).

sFRP1 levels were reduced in breast carcinomas and were maintained in benign breast tumors (34). One study of 70 breast tumors found reduced levels of sFRP1 mRNA to be associated with lymph node metastases and increased mortality (27). In our series of locally advanced colorectal tumors, we found no association between sFRP1 expression levels and the development of lymph node metastases or any other clinical or pathologic characteristics, although this had been suggested by our previous loss of heterozygosity studies (12). This issue may be clarified by analysis of less advanced colorectal cancers and premalignant adenomas for levels of sFRP1 expression. One possibility is that sFRP1 influences tumor progression at an earlier stage of tumor development in colorectal cancer than in breast cancer.

We identified a high frequency (82%) of methylation, comparable with the 95% found by Suzuki et al. (37), but there was not an absolute inverse correlation between methylation and transcription. There could be a number of reasons for this, including heterogeneity in the cancer samples, but the sFRP1 promoter has yet to be characterized fully; therefore, the contributions of each of its three CpG islands to transcriptional control are not known. Thus, our analysis provides an estimate of the frequency of methylation at the sFRP1 locus rather than a direct measurement of modification of a functional site. However, the observation that demethylation leads to expression of sFRP1 in RKO cells demonstrates that there can be a relationship between these processes, and our results show that methylation and transcriptional repression are common events in colorectal cancer (37).

The primary translation product of sFRP1 contains an atypical signal sequence, in which the hydrophobic domain is preceded by a stretch of 15 hydrophilic amino acids. We have identified a common polymorphism, which results in an extra amino acid after codon 13. This insertion was over-represented in the retained alleles of our primary cohort of colorectal tumors with interstitial loss, but secondary analysis of a larger series of tumors failed to show a statistically significant correlation between its presence and the risk of developing colorectal cancer. Additional studies are underway to determine whether this polymorphism has any direct effect on protein activity.

The COOH-terminal domain of sFRP1 is related to netrin 1 (31), a regulator of apoptosis via its interaction with DCC (38), and this netrin-related motif also is found in a range of other proteins where it is thought to mediate protein-protein interactions (39). The identification of sFRP1 as SARP2 (24) demonstrated its potential to promote apoptosis. If this is a response to Wnt occupancy of the N-terminal CRD, it may be modified by differences at the COOH terminus. We have identified alternative splicing, which leads to an extended COOH terminus in a range of tissues, including colon and prostate, and this may influence the role of sFRP1 in tumorigenesis.

According to the model of colorectal cancer progression that has emerged in recent years, APC and β-catenin mutations lead to con-
stuitive stimulation of β-catenin transcription as an early or initiating event, and mutations in the canonical Wnt response pathway upstream of β-catenin would have little effect (16). However, Wnt signaling can act through at least two β-catenin-independent, noncanonical pathways (40, 41). This suggests a model whereby chronic β-catenin signaling leads to a shift in the Wnt response toward these alternative pathways and loss of the antagonist function of sFRP1 hypersensitizes the tumor to Wnt. This loss of stringency in growth factor responses could be an important step in tumor progression.

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