Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer

Although mutation of APC or CTNNB1 (β-catenin) is rare in breast cancer, activation of Wnt signalling is nonetheless thought to play an important role in breast tumorigenesis, and epigenetic silencing of Wnt antagonist genes, including the secreted frizzled-related protein (SFRP) and Dickkopf (DKK) families, has been observed in various tumours. In breast cancer, frequent methylation and silencing of SFRP1 was recently documented; however, altered expression of other Wnt antagonist genes is largely unknown. In the present study, we found frequent methylation of SFRP family genes in breast cancer cell lines (SFRP1, 7 out of 11, 64%; SFRP2, 11 out of 11, 100%; SFRP5, 10 out of 11, 91%) and primary breast tumours (SFRP1, 31 out of 78, 40%; SFRP2, 60 out of 78, 77%; SFRP5, 55 out of 78, 71%). We also observed methylation of DKK1, although less frequently, in cell lines (3 out of 11, 27%) and primary tumours (15 out of 78, 19%). Breast cancer cell lines express various Wnt ligands, and overexpression of SFRPs inhibited cancer cell growth. In addition, overexpression of a β-catenin mutant and depletion of SFRP1 using small interfering RNA synergistically upregulated transcriptional activity of T-cell factor/lymphocyte enhancer factor. Our results confirm the frequent methylation and silencing of Wnt antagonist genes in breast cancer, and suggest that their loss of function contributes to activation of Wnt signalling in breast carcinogenesis.

Keywords: Wnt; SFRP; DKK1; methylation; breast cancer
is a frequent event that has been seen in virtually every tumour type (Ting et al., 2006). When we screened for epigenetically silenced genes in CRC, we found that multiple members of the SFRP family are concurrently methylated at high frequencies among cultured CRC cells and primary CRC specimens (Suzuki et al., 2002). We also found evidence that loss of SFRP function contributes to the activation of Wnt signalling in CRC cells (Suzuki et al., 2004). That SFRP1 is located in a chromosomal region that is frequently deleted in breast cancer (8p12–p11.1) suggests that SFRP1 may also play a tumour suppressor role during mammary tumorigenesis (Ugolini et al., 1999, 2001; Armes et al., 2004).

Consistent with that idea, we have previously found that SFRP1 is methylated in several breast cancer cell lines (Suzuki et al., 2002), and two other groups recently reported frequent SFRP1 methylation in both primary and cultured breast cancer cells (Lo et al., 2006; Veeck et al., 2006). To date, epigenetic silencing of SFRP1 has been identified in a variety of malignancies, including cancers of the bladder (Stoehr et al., 2004), prostate (Lodygin et al., 2005), lung (Fuku et al., 2005) and breast, although abnormalities involving other SFRP family genes are largely unexplored.

We previously showed that SFRP1, SFRP2 and SFRP5 are frequently inactivated in CRC and gastric cancer (GC) (Suzuki et al., 2002; Nojima et al., 2007), and that SFRPs suppress constitutive Wnt signalling when overexpressed in CRC and GC cells. Similarly, Bafico et al. (2004) reported that constitutive Wnt signalling could be suppressed in breast cancer cells by SFRP1 and DKK1. DKK1 also has been shown to be a target of methylation-associated silencing in CRC cells (Aguiera et al., 2006). In the present study, we attempted to characterise the epigenetic abnormalities of Wnt antagonist genes in breast cancer and to determine whether SFRP genes function as tumour suppressors in the breast.

MATERIALS AND METHODS

Cell lines and tissue specimens

Six breast cancer cell lines (MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-468, MDA-MB-157, MDA-MB-453) were purchased from the American Type Culture Collection (Manassas, VA, USA), and five (MCF-7, T-47D, SK-BR-3, MDA-MB-361 and ZR-75-1) were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). The two CRC cell lines (RKO and DKO2) used were described previously (Toyota et al., 2003). All of the cell lines were cultured in the appropriate medium. To analyse restoration of SFRP gene expression, cells were treated with 2 µM 5-aza-2'-deoxycytidine (DAC) (Sigma, St Louis, MO, USA) for 72 h, replacing the drug and medium every 24 h. A total of 38 breast tumour specimens were obtained from Department of Pathology at Johns Hopkins Medicine (Baltimore, MD, USA). In addition, 78 tumour specimens were obtained from Department of Pathology at Johns Hopkins Medicine (Baltimore, MD, USA). Informed consent was obtained from all patients before collection of the specimens. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion, Austin, TX, USA). Total RNA from normal breast tissue from a healthy individual was purchased from BioChain (Hayward, CA, USA).

Reverse transcriptase-PCR

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (RT) (Invitrogen). The integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR was run in a 50-µl volume containing 100 ng of cDNA, 1 × Ex Taq Buffer (TaKaRa, Otsu, Japan), 0.3 mM dNTP, 0.25 µM each primer and 1 U of TaKaRa Ex Taq Hot Start Version (TaKaRa). The PCR protocol entailed 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C; and a 7-min final extension at 72°C. Primer sequences for RT-PCR analysis are listed in Table 1. Real-time RT-PCR was carried out using SYBR Green sequence detection reagents (Applied Biosystems, Foster City, CA, USA) in a 50-µl volume containing 100 ng of cDNA, 25 µl of SYBR Green PCR Master Mix (Applied Biosystems) and 0.2 µM each primer. The PCR protocol entailed 5 min at 95°C and 40 cycles of 30 s at 95°C and 1 min at 60°C. Fluorescent signals were detected using an ABI 7000 Prism 7000 (Applied Biosystems), and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data were analysed using ABI 7000 Prism 7000 SDS Software (Applied Biosystems). Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were used to calculate mRNA copy number in each sample. Ratios of the intensities of the target genes and GAPDH signals were used as a relative measure of the expression level of target genes. Primer sequences for real-time RT-PCR are listed in Table 1.

Methylation analysis

Bisulphite treatment of genomic DNA was performed as described previously (Suzuki et al., 2002). Methylation was then analysed using methylation-specific PCR (MSP) and bisulphite sequencing. PCR was run in a 25-µl volume containing 50 ng bisulphite-treated DNA, 1 × MSP buffer (67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)_2SO₄, 6.7 mM MgCl₂ and 10 mM 2-mercaptopethanol), 1.25 mM dNTP, 0.4 µM each primer and 0.5 U of JumpStart REDTaq DNA Polymerase (Sigma). The PCR protocol for MSP entailed 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C; and a 7-min final extension at 72°C. The PCR protocol for bisulphite sequencing entailed 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C; and a 7-min final extension at 72°C. Amplified bisulphite-sequencing PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 10 clones from each sample were sequenced. Primer sequences are listed in Table 1.

Immunofluorescence microscopy

Cells cultured on chamber slides were washed with PBS and fixed in 4% paraformaldehyde, after which they were incubated with anti-β-catenin monoclonal antibody (BD Transduction Laboratories, San Diego, CA, USA) and stained with anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen). Cells were then examined using an FV300-IX71 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Colony formation assays

Cells (2 × 10⁶ cells) were transfected with 5 µg of one of the pcDNA3.1/His-SFRP vectors or with empty vector using the Cell Line Nucleofector kit V (Amaza, Cologne, Germany) and a Nucleofector 1 electroporation device (Amaza), according to the manufacture’s instructions. The SFRP vectors were prepared as described previously (Suzuki et al., 2004), and an empty vector, pcDNA3.1/HisA (Invitrogen), was used as a control. Cells were plated on 60-mm culture dishes and selected for 14 days with 0.6 mg ml⁻¹ G418, after which colonies were stained with Giemsa and counted using National Institute of Health IMAGE software.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was carried out as described previously (Nojima et al., 2007). Briefly, 2 × 10⁶ cells...
were transfected with one of the SFRP vectors or with an empty vector as described above. Forty-eight hours after transfection, the cells were harvested, fixed with methanol, rehydrated with PBS, treated with 2 μg ml⁻¹ RNAse for 30 min at 37 °C, stained in propidium iodide solution (50 μg ml⁻¹) and analysed using a FACScalibur instrument (Becton Dickinson, San Jose, CA, USA).

Small interfering RNA-mediated knockdown of SFRP1

For small interfering RNA (siRNA)-mediated knockdown of SFRP1, three different oligonucleotide dsRNAs against SFRP1 (siSFRP1) were generated by Dharmacon (Lafayette, CO, USA), after which a mixture of the three was used for transfection. The sequences of the siRNA oligonucleotides are listed in Table 1. A small interfering RNA (siSFRP1) was generated by Dharmacon (Lafayette, CO, USA), and thus viable cells, in a given well.

Table 1  Sequences for primers and siRNA used in this study

| Gene name | Sense | Antisense | Product size (bp) |
|-----------|-------|-----------|------------------|
| RT-PCR    | 5′-CCACGGGATCCAGCTGCAGCTGAGTT-3′ | 5′-CTCAGATTTCAACTCTGTGTACAGG-3′ | 497 |
| SFRP1     | 5′-ATGATGATGACACAGCATATAATG-3′ | 5′-ATGCGCTTGAACCTCTCTGCTCC-3′ | 322 |
| SFRP5     | 5′-CAGATGATGACACAGCATATAATG-3′ | 5′-AGAAGGAGGTTAGTGAAGGAG-3′ | 346 |
| DKK1      | 5′-CTTCTCTTCTCTCTGAGGTGAGTT-3′ | 5′-CTACTGGATGCTGAGTGCTCC-3′ | 404 |
| Wnt1      | 5′-GTCCTGACGACACAGCATATAATG-3′ | 5′-CCTGTTGTGGAAGGTTGATG-3′ | 404 |
| Wnt2      | 5′-TTGAAAACATCTGGTGAGTTGT-3′ | 5′-ACTTACCAACACCTTTGGGAA-3′ | 379 |
| Wnt2B     | 5′-GAGATGATGACACAGCATATAATG-3′ | 5′-TGTGAGTGGAGAGGGGCTTAC-3′ | 338 |
| Wnt3      | 5′-ATGACAGATGACACAGCATATAATG-3′ | 5′-AGGCCTGGAGAGGAGGTATTTG-3′ | 349 |
| Wnt3A     | 5′-GGCATCAAAGATGAGCACTCAGG-3′ | 5′-CATTAGGATGCTGAGTGCTCC-3′ | 404 |
| Wnt4      | 5′-ATGATGATGACACAGCATATAATG-3′ | 5′-TGCGGCTTGAACCTCTCTGCTCC-3′ | 333 |
| Wnt5A     | 5′-TGGAGATGATGACACAGCATATAATG-3′ | 5′-AGCTGTTGGAAGGTTGATG-3′ | 314 |
| Wnt5B     | 5′-AAAGATGATGACACAGCATATAATG-3′ | 5′-ATCCACAAACATCTTTGGGAA-3′ | 355 |
| Wnt7A     | 5′-GAGATGATGACACAGCATATAATG-3′ | 5′-TCTCTTCTTCTGCAGACAGAT-3′ | 310 |
| Wnt7B     | 5′-GAGATGATGACACAGCATATAATG-3′ | 5′-CTCTGTGTGGAAGGTTGATG-3′ | 353 |
| Wnt11     | 5′-CAAGATGATGACACAGCATATAATG-3′ | 5′-TCACTACCTTTCCAGAGGAG-3′ | 364 |
| AXN2      | 5′-TGGAGATGATGACACAGCATATAATG-3′ | 5′-TGTGAGTGGAGAGGGGCTTAC-3′ | 455 |
| MMP7      | 5′-GAGATGATGACACAGCATATAATG-3′ | 5′-AGCCTGTTGGAAGGTTGATG-3′ | 379 |
| GAPDH     | 5′-CTTCTGAAAGATGAGCACTCAGG-3′ | 5′-ATCCATGTTGGAAGGTTGATG-3′ | 130 |
| MMP1      | 5′-CATCTGCTTTAAGATGAGGAGTTG-3′ | 5′-ATCCATGTTGGAAGGTTGATG-3′ | 151 |
| MYD76     | 5′-CTTCTGAAAGATGAGCACTCAGG-3′ | 5′-ATCCATGTTGGAAGGTTGATG-3′ | 126 |
| MYD80     | 5′-CTTCTGAAAGATGAGCACTCAGG-3′ | 5′-ATCCATGTTGGAAGGTTGATG-3′ | 113 |

MSP = methylation-specific PCR; RT = reverse transcriptase; siRNA = small interfering RNA.

Cell viability assay

Proliferation of siRNA transfectants was analysed by measuring the uptake of tritium thymidine in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Transfected cells were seeded into 96-well plates to a density of 5 × 10³ cells per well. After incubation for 48 h, MTT assays were carried out using a Cell Counting kit-8 (Dojindo, Tokyo, Japan), according to the manufacturer’s instructions. The colorimetric read-out in this assay reflects the number of metabolically active mitochondria, and thus viable cells, in a given well.

Analysis of TCF/LEF-mediated transcription

An expression vector encoding a mutant form of β-catenin, pcDNA3.1-β-cateninΔ45, was prepared as described previously (Suzuki et al, 2004). For the combination analysis of mutant β-catenin and siSFRP1, MDA-MB-436 cells were cotransfected with 5 μg of pcDNA3.1-β-cateninΔ45 or empty vector plus 1.5 μg of siSFRP1 or silCONTROL using the Cell Line Nucleofector kit V (Amaza) and a Nucleofector I electroporation device (Amaza), according to the manufacturer’s instructions. Total RNA was extracted 48 h after transfection, and SFRP1 expression was analysed by RT-PCR.

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reporter assays, MDA-MB-436 cells were transfected with 100 ng of pGL3-OT (a TCF/LEF-responsive luciferase reporter plasmid) or pGL3-OF (a negative control plasmid), 100 ng of pcDNA3.1-β-cateninΔ45 or empty vector, 1 ng of pRL-CMV and 100 nM of siSFRP1 or siCONTROL using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

**Statistical analysis**

Statistical analyses were carried out using SPSS software (version 11.0; SPSS Inc., Chicago, IL, USA). Mann–Whitney’s U-test and Fisher’s exact test (two-sided) were used to evaluate the association between methylation of Wnt antagonist genes and clinicopathological features. Values of $P < 0.05$ were considered significant.

**RESULTS**

**Analysis of Wnt antagonist gene expression in breast cancer cell lines**

We previously reported that three of the five SFRP genes (SFRP1, SFRP2 and SFRP5) were frequently methylated and silenced in CRC and GC cells (Suzuki et al., 2002; Nojima et al., 2007). For that reason, in the present study we initially evaluated the expression status of SFRP1, SFRP2 and SFRP5 in a panel of breast cancer cell lines. We found that SFRP1 mRNA was completely absent in four of the 11 cell lines tested (MCF-7, MDA-MB-231, T-47D and SK-BR-3) and was downregulated in one cell line (MDA-MB-453) (Figure 1A). Treating the cells with the DNA methyltransferase (DNMT) inhibitor DAC rapidly restored its expression (Figure 1A).

SFRP2 expression was absent in eight cell lines (MCF-7, MDA-MB-231, MDA-MB-435S, MDA-MB-468, T-47D, SK-BR-3, MDA-MB-453 and ZR-75-1) and downregulated in two (MDA-MB-436 and MDA-MB-361) (Figure 1A), while SFRP5 expression was absent in nine cell lines (MCF-7, MDA-MB-231, MDA-MB-436, T-47D, SK-BR-3, MDA-MB-157, MDA-MB-361, MDA-MB-453 and ZR-75-1) and downregulated in two (MDA-MB-435S and MDA-MB-468) (Figure 1A). 5-Aza-2′-deoxycytidine treatment restored mRNA expression in the majority of the cells in which SFRP2 and/or SFRP5 were downregulated (Figure 1A).

As DKK1 was recently shown to be epigenetically silenced in CRC, we also analysed expression of DKK1 in the breast cancer cells. We found that DKK1 mRNA was significantly downregulated in two cell lines (T-47D and MDA-MB-453), and the expression was restored by DAC treatment (Figure 1A). In contrast to cancer cells, all Wnt antagonist genes were expressed in normal breast tissue (Figure 1A).

**Analysis of Wnt antagonist gene methylation in breast cancer cell lines**

We next used MSP to analyse the methylation status of Wnt antagonist genes. A CRC cell line (RKO) in which all of the Wnt antagonist genes were methylated served as a positive control, while another CRC cell line (DKO2) in which the DNMT1 and DNMT3B loci were genetically disrupted served as a negative control.
control (Figure 1B). We observed SFRP1 methylation in 7 of the 11 (64%) breast cancer cell lines tested (MCF-7, MDA-MB-231, T-47D, SK-BR-3, MDA-MB-361, MDA-MB-453 and ZR-75-1) (Figure 1B). Of those, expression of SFRP1 mRNA was down-regulated in five, as described above. We also detected SFRP1 methylation in two cell lines in which basal expression of the gene remained intact (MDA-MB-361 and ZR-75-1) (Figure 1A and B). As both methylated and unmethylated SFRP1 was detected in these cells, we suggest that the mRNA was likely transcribed from the unmethylated allele.

SFRP2 methylation was detected in all 11 cell lines (100%), while SFRP5 methylation was detected in 10 (91%) (Figure 1B). In general, we observed a good correlation between methylation and the expression status of SFRP2 and SFRP5, although there were some exceptions. For example, some methylation of SFRP2 was detected in MDA-MB-157 cells (Figure 1B), but they showed a substantial amount of basal mRNA expression (Figure 1A), which was likely transcribed from the unmethylated allele. Conversely, SK-BR-3 cells showed no SFRP5 methylation but did not express any mRNA. Since DAC treatment did not restore SFRP5 expression in SK-BR-3 cells, a different mechanism may be responsible for its silencing.

Methylation of DKK1 was detected in three cell lines (T-47D, MDA-MB-361 and MDA-MB-453) (Figure 1B), although none of the cells showed complete methylation of DKK1, and MSP revealed both methylated and unmethylated DNA (Figure 1B). These results are consistent with the observation that two cell lines showed significant downregulation of DKK1 expression, but the silencing was apparently incomplete, as low levels of expression were detectable with RT-PCR (Figure 1A). In contrast to cancer cell lines, no methylation of Wnt antagonist genes was detected in normal breast tissue (Figure 1B).

To analyse the methylation status in more detail, we carried out bisulphite sequencing in selected cell lines. Sequence analysis revealed that the CpG islands of SFRP1, SFRP2 and SFRP5 are extensively methylated in MCF-7 cells (Figure 2A – C). By contrast, almost no methylation of SFRP1 was detected in MDA-MB-436 cells (Figure 2A), which is consistent with the MSP results and the finding that these cells express SFRP1 (Figure 1). We also analysed SFRP2 in MDA-MB-436 cells, where both methylated and unmethylated DNA was detected by MSP. Bisulphite sequencing revealed a heterogeneous pattern of SFRP2 methylation (Figure 2B), and the incomplete silencing of SFRP2 in this cell line likely reflects the low-density methylation of the gene.

Figure 2  Bisulphite-sequencing analysis of SFRP gene methylation. (A) Bisulphite sequencing of SFRP1 in the indicated breast cancer cell lines and normal breast tissue. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The locations of MSP primer sites are shown by arrows on the top. The location of each CpG site relative to the transcription start site is shown below. (B, C) Bisulphite sequencing of SFRP2 (B) and SFRP5 (C). CpG sites are represented as in panel A.
similar heterogeneous methylation pattern was observed for SFRP5 in MDA-MB-468 cells, where partial methylation was detected with MSP (Figures 1A and 2C). Normal breast tissue showed little or no methylation of SFRP1, SFRP2 or SFRP5 (Figure 2A–C).

Analysis of Wnt antagonist gene methylation in primary breast tumours

Our finding of epigenetic silencing of SFRP genes and DKK1 in breast cancer cell lines prompted us to determine the extent to which these Wnt antagonist genes are also aberrantly methylated in primary breast tumours. We first analysed SFRP methylation in 38 breast tumour specimens and frequently found SFRP methylation in these tumours (SFRP1, 12 out of 38, 32%; SFRP2, 28 out of 38, 74%; SFRP5, 22 out of 38, 58%). We then examined the 78 specimens from Japanese breast cancer patients and found the SFRP genes to be methylated at similar frequencies (SFRP1, 31 out of 78, 40%; SFRP2, 60 out of 78, 77%; SFRP5, 55 out of 78, 71%). On the other hand, methylation of DKK1 was detected in only 15 (19%) of the 78 tumours. We also obtained samples of adjacent non-tumourous breast tissue from 20 of the 78 Japanese patients. In general, methylation of SFRPs and DKK1 was tumour-specific or tumour-predominant, but weak methylation of SFRP genes was observed in some non-tumourous breast tissues (representative results are shown in Figure 3). This may have been caused by field defects.

The clinicopathological features were available from the Japanese patients, but there were no significant correlations between the methylation status of the Wnt antagonist genes and age, pT status, pN status, pM status, oestrogen receptor status or HER2 status (Table 2).

SFRPs suppress breast cancer cell growth

To determine whether SFRP genes function as tumour suppressors in breast cancer, we next analysed Wnt signalling in breast cancer cells. We first used RT-PCR to test for the expression of Wnt ligands. We found that all of the cell lines expressed at least 6 of the 11 Wnt ligand genes tested (Figure 4). Subsequent immunofluorescence analysis of the intracellular distribution of β-catenin in seven breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-468, T-47D and SK-BR-3) revealed nuclear accumulation of β-catenin in four cell lines (MCF-7, MDA-MB-231, T-47D and SK-BR-3) in which SFRP1, SFRP2 and SFRP5 were silenced (Supplementary Figure 1). No mutation of APC

![Figure 3](https://www.nature.com/doi/abs/10.1038/nature08683)

**Figure 3** Analysis of methylation of Wnt antagonist genes in primary breast cancers. Shown are representative results of MSP analysis of SFRP1, SFRP2, SFRP5 and DKK1 in primary breast cancer tissues (T) and adjacent normal breast tissues from the same patients (N).

| Table 2 | Clinicopathological features of breast cancer with or without Wnt antagonist genes methylation |
|---------|--------------------------------------------------------------------------------------------------|
|         | SFRP1 | SFRP2 | SFRP5 | DKK1 |
| **n**   | U | M | P-value | U | M | P-value | U | M | P-value | U | M | P-value |
| **Total** | 78 | 47 | 31 | 18 | 60 | 23 | 55 | 63 | 15 | 63 | 15 |
| **Age (years, mean ± s.d.)** | 53.66 ± 11.01 | 52.22 ± 11.97 | 53.56 ± 12.03 | 52.95 ± 11.24 | 52.78 ± 8.88 | 53.22 ± 12.31 | 52.46 ± 11.43 | 55.73 ± 10.96 |
| **pT category** | | | | | | | | | | | | |
| pTis | 1 | 0 | 1 | 0.796 | 0 | 1 | 0.629 | 0 | 1 | 0.860 | 1 | 0 |
| pT1 | 17 | 12 | 5 | 4 | 13 | 6 | 11 | 13 | 4 |
| pT2 | 41 | 25 | 16 | 11 | 30 | 10 | 31 | 32 | 9 |
| pT3 | 8 | 4 | 4 | 1 | 7 | 5 | 3 | 8 | 0 |
| pT4 | 7 | 5 | 2 | 1 | 6 | 7 | 0 | | |
| **pN category** | | | | | | | | | | | | |
| pN0 | 35 | 20 | 15 | 0.809 | 9 | 26 | 0.678 | 7 | 28 | 0.125 | 29 | 6 |
| pN1 | 28 | 20 | 8 | 6 | 22 | 11 | 17 | 21 | 7 |
| pN2 | 6 | 2 | 4 | 2 | 4 | 1 | 5 | 6 | 0 |
| pN3 | 3 | 2 | 1 | 0 | 3 | 2 | 1 | 3 | 0 |
| **pM category** | | | | | | | | | | | | |
| pM0 | 66 | 40 | 26 | 1.000 | 17 | 49 | 0.325 | 20 | 46 | 0.664 | 53 | 13 |
| pM1 | 6 | 4 | 2 | 0 | 6 | 1 | 5 | 6 | 0 |
| **ER** | | | | | | | | | | | | |
| Negative | 15 | 10 | 5 | 0.517 | 6 | 9 | 0.292 | 6 | 9 | 0.128 | 14 | 1 |
| Positive | 26 | 13 | 13 | 5 | 21 | 4 | 22 | 19 | 7 |
| **HER2** | | | | | | | | | | | | |
| Negative | 24 | 15 | 9 | 0.353 | 9 | 15 | 0.086 | 5 | 19 | 0.714 | 19 | 5 |
| Positive | 18 | 8 | 10 | 2 | 16 | 5 | 13 | 15 | 3 |
| **Histology** | | | | | | | | | | | | |
| Ductal carcinoma in situ | 4 | 3 | 1 | 2 | 2 | 1 | 3 | 4 | 0 |
| Invasive ductal carcinoma | 63 | 37 | 26 | 11 | 52 | 18 | 45 | 51 | 12 |
| Lobular carcinoma in situ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Invasive lobular carcinoma | 2 | 1 | 1 | 0 | 2 | 1 | 1 | 1 | 1 |
| Others | 7 | 6 | 1 | 4 | 3 | 3 | 4 | 5 | 2 |

ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2.
whether loss of SFRP function leads to upregulated Wnt signalling. Although transient transfection of siSFRP1 reduced levels of SFRP1 mRNA by approximately 90% (Figure 6A and B), no upregulation in TCF/LEF transcriptional activity was observed (Figure 6C), and expression of TCF/LEF target genes was unchanged (Figure 6A, D and E).

By contrast, transient transfection of a mutant form of β-catenin(A5S) induced a 10-fold increase of pG3.OT reporter activity (Figure 6C) and upregulated the mRNA expression of AXIN2 and MMP7, two TCF/LEF target genes (Figure 6A, D and E). Furthermore, reporter assays and analysis of target gene expression revealed that the combination of SFRP1 depletion and β-catenin(A5S) overexpression acted synergistically to upregulate TCF/LEF transcription (Figure 6A, C–E).

**DISCUSSION**

Compelling evidence suggests that activation of Wnt signalling plays an important role in breast cancer. Immunohistochemical staining carried out by several groups has demonstrated elevated levels of nuclear and/or cytoplasmic β-catenin in breast tumours with significant frequency (Lin et al., 2000; Ryo et al., 2001; Chung et al., 2004). Lin et al. (2000) also showed that nuclear and/or cytoplasmic staining of β-catenin correlated with elevated cyclin D1, which is one of the known targets of β-catenin/TCF transcription. However, mutation of APC, CTNNB1 or AXIN is rare in breast cancer, and thus the mechanism of Wnt signal activation in this disease is not fully understood.

On the basis of their expression array analysis of chromosome 8p11–21 genes, Ugolini et al. (1999) reported that expression of SFRP1 mRNA is frequently diminished or lost in breast carcinomas, and they also found that loss of SFRP1 occurs in more than 80% of invasive breast carcinomas, although not in the medullary type (Ugolini et al., 2001). Using immunohistochemical analysis, Kloppocki et al. (2004) found that expression of SFRP1 protein was absent in 46% of invasive breast tumours and in 43% of carcinoma in situ. In addition, two groups recently reported frequent SFRP1 methylation in primary breast carcinomas (Lo et al., 2006; Veeck et al., 2006), and Bafico et al. (2004) clearly demonstrated a novel autocrine mechanism leading to constitutive Wnt signalling in breast cancer, which could be suppressed by SFRP1 and DKK1. Taken together, these results suggest that loss of SFRP1 function is a key mechanism by which Wnt signalling is activated in breast cancer.

To date, four SFRP family genes (SFRP1, SFRP2, SFRP4 and SFRP5) and two DKK family genes (DKK1 and DKK3) have been identified as targets of epigenetic silencing in human tumours (Suzuki et al., 2002; Roman-Gomez et al., 2004; Lodygin et al., 2005; Aguilera et al., 2006; Niehrs, 2006). Among them, we showed that loss of SFRP1, SFRP2 and SFRP5 contributes to Wnt signal activation in both CRC and GC. Dickkopf proteins bind with LRPs and inhibit Wnt signalling by preventing Wnt and Fz from forming a ternary complex with LRPs. However, among the DKK family, DKK3 is the most divergent, as it neither binds LRPs nor significantly affects Wnt signalling (Niehrs, 2006). We therefore analysed methylation and expression status of SFRP1, SFRP2, SFRP5 and DKK1. SFRP1, SFRP2, SFRP5, and DKK1 were methylated in 64, 100, 91, and 27% of the breast cancer cell lines tested, respectively. In primary breast tumours, methylation frequencies of SFRP1, SFRP2, SFRP5 and DKK1 were 40, 77, 71 and 19%, respectively. Although the frequency of SFRP1 methylation in our samples was somewhat lower than those reported by Lo et al. (2006) and Veeck et al. (2006), we also found that a substantial number of breast tumours harbour SFRP1 methylation. In addition, we found that SFRP2 and SFRP5 are methylated in both cultured breast cancer cells and primary breast cancers at quite high frequencies. In contrast to SFRP genes, however, the frequency of DKK1 methylation in breast cancer was relatively limited.
To examine the activity of Wnt signalling in breast cancer, we evaluated the expression status of the Wnt ligands in the cell lines. We previously found that at least 3 of the 11 Wnt ligands were expressed in CRC cell lines (Suzuki et al., 2004), and even more Wnt ligands were expressed in breast cancer cell lines. Using immunofluorescence analysis, we observed β-catenin to be present in the nuclei of four of seven breast cell lines in which multiple SFRP genes were methylated and silenced. Ectopic expression of SFRP in breast cancer cells suppressed colony formation and induced apoptosis. Conversely, SFRP1 knockdown enhanced breast cancer cell growth. Thus, loss of Wnt antagonists, particularly SFRPs, appears to contribute to Wnt signal activation in breast cancer.

On the other hand, we found that none of the breast cancer cell lines tested showed upregulated TCF/LEF transcriptional activity, even when they showed a loss of Wnt antagonist genes and nuclear accumulation of β-catenin, which is consistent with our earlier findings in GC cells. Although it is well known that mutation in APC, CTNNB1 and AXIN is infrequent in GC, we found that the majority of GC cells showed activation and nuclear accumulation of β-catenin and methylation of multiple SFRP genes (Nojima et al., 2007). Among those GC cells, only a few cell lines in which APC or CTNNB1 was mutated showed significant upregulation of TCF/LEF transcriptional activity (Nojima et al., 2007). It is thus possible that the TCF/LEF reporter assay used may not be sufficiently sensitive to detect the weak or moderate activation of canonical Wnt signalling.

Alternatively, the tumour-suppressive function of Wnt antagonists may be independent of the Wnt/β-catenin pathway. To clarify the functional role of SFRP inactivation in breast cancer, we used siRNA to disrupt endogenous SFRP1 expression. Because none of the breast cancer cell lines presented aberrant TCF/LEF transcription, we used an expression vector encoding a β-catenin mutant to boost Wnt signalling. We found that the TCF/LEF transcriptional activity induced by the mutant β-catenin was synergistically upregulated when combined with SFRP1 depletion, which is consistent with the recent observation by Fukui et al. (2005). Using non-small-cell lung carcinoma cells in which SFRP1 was methylated, they showed that when SFRP1 was cotransfected with mutant β-catenin, SFRP1 could attenuate TCF/LEF activity induced by exogenous β-catenin. Our results demonstrate the potential of endogenous SFRP1 to inhibit the Wnt/β-catenin pathway in breast cancer cells.

In summary, we have shown that epigenetic silencing of multiple Wnt antagonist genes is a common event in breast cancer. In particular, we found that SFRP2 and SFRP5 are methylated at even higher frequencies than SFRP1. Taken together, our findings indicate that the majority of breast tumours harbour methylation of at least one Wnt antagonist gene, and support the hypothesis that epigenetic silencing of Wnt antagonist genes is a major cause of constitutive Wnt signalling in breast cancer. Our data also suggest that methylation of a set of multiple Wnt antagonist genes may represent a useful marker in breast cancer.

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