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Methylation-associated silencing of SFRP1 with an 8p11-12 amplification inhibits canonical and non-canonical WNT pathways in breast cancers

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Abstract

Recently we analyzed the 8p11-12 genomic region for copy number and gene expression changes in a panel of human breast cancer cell lines and primary specimens. We found that *SFRP1* (*Secreted frizzled related protein 1*) is frequently under expressed even in breast tumors with copy number increases in this genomic region. *SFRP1* encodes a WNT signaling antagonist, and plays a role in the development of multiple solid tumor types. In this study, we analyzed methylation-associated silencing of the *SFRP1* gene in breast cancer cells with the 8p11-12 amplicon, and investigated the tumor suppressor properties of *SFRP1* in breast cancer cells. *SFRP1* expression was markedly reduced in both the breast cancer cell lines and primary tumor specimens relative to normal primary human mammary epithelial cells even when *SFRP1* is amplified. Suppression of *SFRP1* expression in breast cancer cells with an *SFRP1* gene amplification is associated with *SFRP1* promoter methylation. Furthermore, restoration of *SFRP1* expression suppressed the growth of breast cancer cells in monolayer, and inhibited anchorage independent growth. We also examined the relationship between the silencing of *SFRP1* gene and WNT signaling in breast cancer. Ectopic *SFRP1* expression in breast cancer cells suppressed both canonical and non-canonical WNT signaling pathways, and *SFRP1* expression was negatively associated with the expression of a subset of WNT responsive genes including *RET* and *MSX2*. Thus, down-regulation of *SFRP1* can be triggered by epigenetic and/or genetic events and may contribute to the tumorigenesis of human breast cancer through both canonical and non-canonical WNT signaling pathways.

Keywords

SFRP1; amplification; DNA methylation; Wnt pathway; Breast Cancer

Introduction

Cancer cells have diverse biological capabilities that are conferred by numerous genetic and epigenetic aberrations. Genetic aberrations include chromosome number changes, gene translocations, gene amplifications, mutations and deletions 1. Epigenetic abnormalities involve DNA methylation, histone modification, and chromatin remodeling 2, 3. Given that genetic and epigenetic aberrations can interact directly and indirectly during tumorigenesis

3, defining these interactions will provide new insights into cancer development and will provide potential new avenues for therapeutic development.

In human breast cancer (HBC), the 8p11-12 region is a frequent target of genetic alterations such as chromosomal breaks, loss and DNA amplification. Amplification of 8p11-12 occurs in approximately 15% of HBC. This region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients 4-7. A recent study also demonstrates that the region of recurrent amplification at 8p11-12 was reduced in copy number in a subset of HBC, and this genetic loss associates with poor progress 8. Thus, the 8p11-12 region may contain multiple candidate oncogenes and tumor suppressor genes relevant to human breast cancer. Recently, we analyzed the 8p11-12 genomic region for copy number and gene expression changes in a panel of human breast cancer cell lines and primary tumor specimens. We identified 21 genes in this region that are over expressed when their copy number is increased 4. Furthermore, we directly tested the transforming function of these genes in human mammary epithelial cells. From these experiments, we identified several genes including *LSM1*, *BAG4* and *C8orf4* (*TC-1*) have the oncogenic properties *in vitro* 4. However, several genes within 8p11-12 region, including *SFRP1* (*Secreted frizzled related protein 1*) were found to be under expressed even in tumors with gene copy number increases.

SFRP1 protein is homologous to the extracellular cysteine-rich-domain of the WNT receptor Frizzled, but lacks the transmembrane and intracellular domains 9. SFRP1 protein functions as a negative regulator of WNT signaling by sequestering WNT protein and by heterodimerizing with Frizzled to form a non-functional receptor complex 10. The two known WNT signaling pathways play essential roles in the regulation of cell proliferation, patterning and fate determination during normal developmental processes 11. The canonical pathway operates by stabilizing beta-catenin, whereas the non-canonical pathways do not require beta-catenin signaling.

Recently accumulated data demonstrate that loss of various antagonists including SFRP1 through promoter methylation, leads to overactivation of WNT signaling pathways, promoting tumorigenesis in human mammary tissues 12-19. Ugolini *et al.* demonstrated that SFRP1 mRNA expression is lost in more than 80% of invasive breast carcinomas 17. Bafico *et al.* reported that constitutive Wnt signalling could be suppressed in breast cancer cells by SFRP1 18. Schlange *et al.* show that SFRP1 blocks proliferation of many breast tumor cell lines through interference with pathway activation that is presumably driven by endogenous WNT ligands 19. One study revealed that SFRP1 promoter methylation was detected in 61% of primary breast cancers. Kaplan-Meier analysis showed SFRP1 gene hypermethylation was associated with a shorter overall survival of patients with invasive breast cancer 13. In addition, Cowling *et al.* demonstrated that c-Myc transforms human mammary epithelial cells through repression of the WNT inhibitors DKK1 and SFRP1, and suggests a positive feedback loop for the activation of the c-Myc and WNT pathways in breast cancer 20.

In this study, we analyzed methylation-associated silencing of the *SFRP1* gene in breast cancer cells with the 8p11-12 amplicon. We demonstrate that suppression of SFRP1 expression in breast cancer cells with an *SFRP1* gene amplification was attributable to an epigenetic mechanism. Furthermore, cells with methylation-induced silencing of the *SFRP1* gene exhibited reduced growth kinetics after restoration of SFRP1 expression. Our reporter assays demonstrated that re-expression of SFRP1 in breast cancer cells suppressed both the canonical and non-canonical WNT signaling pathways. In addition, SFRP1 expression was negatively associated with a subset of WNT responsive genes including known oncogenes *RET* and other growth regulators such as *MSX2*. Thus, SFRP1 has growth-suppressive

properties that may affect both canonical and non-canonical WNT signaling pathways in human breast cancer.

Materials and Methods

Cell lines and tumor specimens

Culture of the SUM series of HBC cell lines and the immortalized, nontransformed mammary epithelial cell line, MCF10A, have been described in detail previously 21. Primary human breast cancer specimens were obtained from Asterand Inc. (Detroit, MI).

Quantitative RT-PCR reactions

Total RNA was prepared from HBC cell lines, the MCF10A cell line and tumor specimens by standard methods 4, 22. For quantitative RT-PCR reactions, RNA was converted into cDNA via a reverse transcription reaction using random hexamer primers. SFRP1 primer set was ordered from Invitrogen (Carlsbad, CA, USA). A GAPDH primer set was used as a control. Quantitative RT-PCR was done in 25 μ L reactions, in 96-well plates, using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Interpretation of relative expression data was calculated as described by Livak and Schmittgen 23.

Methylation-specific PCR

Genomic DNA from cell lines or primary breast cancer tissues was isolated using standard techniques as described 22. Tissue and cell line DNAs were treated with sodium bisulfite using a CpGenome Fast DNA Modification Kit (Millipore, Billerica, MA), purified, and subjected to methylation-specific PCR (MSP) based on the protocol described previously by Herman *et al.* 24. Sodium bisulfite-treated DNA was amplified using primers specific either for the methylated or for the unmethylated DNA under the conditions as described 25.

Primer sequences for the SFRP1 promoter are 5'-

GAGTTAGTGTGTGTGTTTGTGTTTGT (forward) and 5'-

CCCAACATTACCCAACCTCCACAACCA (reverse) for unmethylated reactions, and 5'-

GTGTCGCGCGTTCGTCGTTTCGC (forward) and 5'-

AACGTTACCCGACTCCGCGACCG (reverse) for methylated reactions. The PCR

products were resolved by electrophoresis in a 2% agarose gel, and the ethidium bromide-stained PCR products were imaged with the Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

Transient transfection and colony formation assays

To generate the SFRP1 expression construct pcDNA-GW-SFRP1, we first created an entry clone containing the full-length SFRP1 using the pENTR directional TOPO cloning kit (Invitrogen, Carlsbad, CA, USA). After we generated the entry clone, we performed the LR recombination reaction to transfer the gene into the pcDNA-DEST47 vector to create the expression clone. The construct was sequenced to ensure that the sequences and orientation were correct. For transient transfection experiments, SUM-44 and SUM-52 cells (5×10^5) were plated in six-well plates 24 hours before transfection. FuGENE HD Transfection Reagent (Roche, Mannheim, Germany) was used to mediate transfection using 2.0 μ g pcDNA-GW-SFRP1 construct or 2.0 μ g control pcDNA-GW-CAT vector according to the manufacturer's protocol. The cells were selected by 400 μ g/ml (SUM-44) or 200 μ g/ml (SUM-52) G418. Colonies were stained with the HEMA3 stain set solution (Fisher Scientific, USA) and were counted 4 weeks after the transfection.

Lentivirus construction and transduction of cells

To generate a lentiviral expression construct (pLenti6-SFRP1) containing the *SFRP1* gene, we performed the LR recombination reaction to transfer the *SFRP1* gene from pENTR vector into the pLenti6/V5-DEST vector to create the expression clone. Production of lentivirus was achieved by cotransfecting the 293FT cell line with the pLenti expression construct and the optimized packaging mix (Invitrogen, Carlsbad, CA, USA). The human breast cancer cell lines SUM-44 and SUM-52 were transduced with lentivirus. Control infections with pLenti6-LacZ virus were performed in parallel with pLenti6-SFRP1 infections. Selection began 48 hours after infection in growth medium with 10 µg/mL blasticidin. Upon confluence, selected cells were passaged and serially cultured.

Soft agar assays

Soft agar assays were performed as previously described [26]. Briefly, dishes were coated with a 1:1 mix of the appropriate 2x medium for the cell line being studied and 1% Bactoagar. On to this was layered 1 ml of a 0.3% agarose cell suspension with 1×10^5 cells. Cells were fed 3 times /week for 3 weeks, stained with 500 µg/ml p-iodonitrotetrazolium violet (Sigma, St Louis, MO, USA) overnight, photographed, and counted on an Accucount 1000 (Fisher Scientific, USA).

Reporter assays

TCF/LEF, AP1 and NFAT reporter assays were performed in SUM-44 and SUM-52 cells stably expressing SFRP1 (pLenti6-SFRP1) or LacZ control (pLenti6-LacZ) using Cignal Reporter Assay Kits (SABiosciences, Frederick, MD, USA). Lipofectamine transfection reagent (Invitrogen) was used to transfect the reporter constructs, negative and positive control into 2×10^4 cells in 96-well plates. After 12-16 hours of transfection, the cells were changed into complete growth medium. After 2 days, luciferase activity was measured using the dual luciferase assay system (Promega, Madison, WI). The relative reporter activity values were expressed as arbitrary units per µg protein measured using the Bradford assay protocol. Transfection efficiency was determined by monitoring GFP expression in transfected cells by fluorescence microscopy using a Nikon Eclipse TE2000-U microscope. Experiments were repeatedly done in triplicate, and the standard deviation was calculated for each experiment.

Microarray and bioinformatics analysis

RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). For Affymetrix microarray experiments, a linear amplification protocol was used and consisted of one round of double-strand cDNA synthesis followed by *in vitro* transcription. The labeled cRNA (10 µg total) was fragmented and used in the hybridization reaction to GeneChip probe array HG U133A according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Each sample was run and the resulting cel files created by Affymetrix MAS5 were analyzed with the open source R Statistical Environment (www.r-project.org) using libraries from the Bioconductor Project (www.bioconductor.org). Quantile normalized gene expression levels were summarized using a sequence specific expression model provided by the Bioconductor library "germa" [27]. Discriminant analysis of SFRP1 under expressing cells relative to SFRP1 expressing cells was performed using a moderated t-test implemented in the Bioconductor library "limma" [28].

Probability values were corrected with the use of the false discovery rate method (FDR). The association of *SFRP1* under expression with WNT/β-catenin-responsive gene expression was determined using Pearson correlation.

Results

SFRP1 expression is markedly reduced in breast cancer even when the SFRP1 gene is amplified

We recently mapped the 8p11-12 amplicon in a panel of human breast cancer cell lines and primary cancer specimens with quantitative genomic PCR and array comparative genomic hybridization (CGH). We demonstrated that three human breast cancer cell lines SUM-44, SUM-52 and SUM-225, have overlapping amplifications in the 8p11-12 region. The SUM-44 cells have a single focal region of amplification, whereas in SUM-52 cells there are two distinct peaks of amplification, and the SUM-225 cells have two small but high-level sub-regions of amplification 4·7·29. The *SFRP1* gene, which encodes an antagonist of the WNT receptors, is located at chromosome 8p11 region. As shown in Figure 1A and table 1, our array CGH, together with previous chromosome CGH, demonstrated that two of 11 SUM breast cancer cell lines, SUM-44 and SUM-52 cells, have *SFRP1* gene amplification (> 2 fold increase in array CGH). Furthermore, array CGH detected that three specimens, 10173, 6617 and 69, of twenty two primary breast cancers have an *SFRP1* gene amplification (Figure 1A· Table 1). To assess the level of *SFRP1* expression in breast cancer cells, we performed mRNA expression analysis in our panel of breast cancer cell lines, primary breast cancer specimens and in the immortalized, nontransformed mammary epithelial cell line, MCF10A. *SFRP1* expression levels were measured using both Affymetrix GeneChip microarrays and/or Q-RT-PCR and were subsequently compared to control MCF10A cells. The results of the microarray assays demonstrated that *SFRP1* expression is markedly reduced (>4 fold decrease) in eight breast cancer cell lines including SUM-44 and SUM-52, both of which have *SFRP1* gene amplification (Table 1). These results were verified by RT-PCR (supplemental data). In addition, *SFRP1* is down regulated when compared with normal breast tissue (>4 fold decrease) in 72.7% (17/22) of primary breast cancer specimens (Table 1 and supplemental data) 4. These seventeen specimens with decreased *SFRP1* include the three specimens, 10173, 6617 and 69, that have an *SFRP1* gene amplification. Thus, the *SFRP1* expression was markedly reduced in multiple cell lines and in nearly 70% of our panel of breast cancer samples even when the *SFRP1* gene is amplified.

To obtain further support for a potential involvement of the *SFRP1* gene in breast cancer, we searched gene expression data sets from the cancer gene microarray meta-analysis public database, Oncomine 3.0. Using this data-mining approach, we found significant under expression of *SFRP1* in 7 datasets of 5 independent studies on primary breast cancer specimens. These studies comprised a total of 235 tumor specimens, 7 benign breast specimens and 29 normal breast specimens 31-35. In this analysis, *SFRP1* was clearly under expressed in breast cancer specimens compared with normal breast specimens (Figure 1). Taken together, these results show that *SFRP1* expression is markedly reduced in breast cancer.

Mechanisms underlying the down regulation of *SFRP1* expression in breast cancer with *SFRP1* gene amplification

In order to unravel whether suppression of *SFRP1* expression in breast cancer cells, particularly cells with *SFRP1* gene amplification, is attributable to epigenetic mechanisms, we first analyzed the methylation status of the *SFRP1* promoter in our panel of 10 breast cancer cell lines by methylation-specific polymerase chain reaction (MSP). Only methylated DNA molecules were detected in two cell lines, SUM-44 and SUM-52, both of which have *SFRP1* gene amplification. Furthermore, both methylated and unmethylated DNA molecules were detected in SUM-229, yet only unmethylated DNA molecules were detected in all other cancer cell lines and in control MCF10A cells (Figure 2A). Nine primary breast cancer

specimens with dramatically decreased *SFRP1* mRNA (>16 fold decrease) were chosen in order to analyze the methylation status of the *SFRP1* promoter. Methylated and unmethylated DNA molecules were detected in six specimens, two (10173 and 6617) of which have *SFRP1* gene amplification (Figure 2B).

To further support the hypothesis that aberrant *SFRP1* methylation is the cause of *SFRP1* silencing in breast cancer cell lines with *SFRP1* gene amplification, we treated two representative breast cancer cell lines, SUM-44 and SUM-52, and the control cell line MCF10A, with the DNA methyltransferase inhibitor 5-aza-deoxycytidine (5-aza-dC). These cells were collected after 3 days of treatment with 1 μ M 5-aza-dC and *SFRP1* expression was analysed by Q-RT-PCR (Figure 2C). We found that expression of *SFRP1* was restored in both SUM-44 and SUM-52 cells, indicating that DNA methylation is the predominant epigenetic mechanism for *SFRP1* gene silencing in these cells.

SFRP1 has tumor suppressor properties in breast cancer cells

Given that *SFRP1* was down regulated in multiple cell lines and in approximately 70% of breast tumor tissue samples, we addressed the question of whether SFRP1 possesses tumor suppressor properties. First we performed a colony formation assay to test the growth-inhibitory effect of exogenously expressed SFRP1. After transfection of the *SFRP1* expression plasmid, pcDNA-GW-SFRP1, into SUM-44 and SUM-52 cells, we selected transfected cells with G418 and counted the surviving colonies after 4 weeks. *SFRP1* expression levels were detected in parallel transfected cells by RT-PCR (Figure 3A). SFRP1 transfection showed suppression of colony formation in both the SUM-44 and SUM-52 cells (Figure 3B). The results were most striking for SUM-52 cells in which SFRP1 expression inhibited colony formation by ~ 60% (Figure 3C). These results indicate that SFRP1 has growth-suppressive activity in human breast cancer.

To further measure the contribution of SFRP1 repression to the transformed phenotype of breast cancer cell lines, we exogenously and stably expressed SFRP1 in SUM-44 and SUM-52 cells using a lentiviral expression system and performed a soft agar assay to measure anchorage independent cell growth. The protein levels of SFRP1 in SFRP1-transduced SUM-44 and SUM-52 cells were measured with western blot (Figure 3D). We found that both SUM-44 and SUM-52 cells transduced with control lentivirus (pLenti6-LacZ) were able to grow into robust colonies in soft agar. SFRP1 expression inhibited soft agar colony growth in both cell lines (Figure 3E). Therefore, restoration of SFRP1 expression suppressed the growth of breast cancer cells in monolayer, and inhibited anchorage independent growth.

Suppression of both canonical and non-canonical WNT signaling pathways by ectopic SFRP1 expression in breast cancer cells

Given that SFRP1 is a WNT antagonist and that the loss of SFRP1 expression is reportedly associated with activation of the WNT signaling pathway in multiple cancer types, we next examined the relationship between the silencing of SFRP1 gene and WNT signaling in breast cancer. WNT signals are transduced through at least three distinct intracellular pathways, including the canonical WNT/ Beta-catenin signaling pathway and the non-canonical WNT/Ca²⁺ (Calcium) and WNT/PCP (planar cell polarity) pathways. To examine the effects of SFRP1 on both canonical and non-canonical WNT signaling pathways, we used a dual-luciferase reporter system that measures transcription from a promoter under the control of tandem repeats of one of three transcription factor elements, TCF, NFAT or AP1 binding sites, which mediate the transcriptional response to activation of beta-catenin, NAFT /calcium pathway or JNK/PCP pathway, respectively. Two stably expressed SFRP1 cell lines, SUM-44-SFRP1, SUM-52-SFRP1 and their vector control cells were transfected

with the firefly luciferase reporter and the CMV control constructs of a Cignal Report Assay Kit. As shown in figure 4, ectopic expression of *SFRP1* in SUM-44 and SUM-52 cells inhibited TCF reporter activity. Furthermore, NFAT and AP1 reporter activity were also inhibited by re-expression of *SFRP1* in these cells. The effects were most remarkable for SUM-52 cells where *SFRP1* over expression down regulated AP1, NFAT and TCF/LEF-driven reporter activity by ~3-fold, ~2-fold and ~6-fold respectively. Co-transfection with a CMV-driven GFP plasmid construct showed that transfection efficiency and constitutive expression from cmv-driven Renilla luciferase was equal for each *SFRP1* transfected cell line compared to their corresponding control (data not shown). Thus, ectopic *SFRP1* expression in breast cancer cells suppresses both canonical and non-canonical WNT signaling pathways.

We next examined the relationship between silencing of the *SFRP1* gene and WNT/ Beta-catenin responsive gene expression in breast cancer samples. This analysis included the Affymetrix-based gene expression profiles from our SUM human breast cancer cell lines and from published data of 130 primary breast cancer samples 8-36. The Affymetrix 133A array includes 127 probe sets for 62 WNT/ Beta-catenin responsive genes. These target genes were identified from a review of the literature, and from those listed on the Nusse laboratory website (<http://www.stanford.edu/~rnusse/wntwindow.html>). Pearson correlation was calculated for the expression of each gene relative to *SFRP1*. We found *SFRP1* expression was negatively correlated with several known oncogenes including *RET* and *CCND1*, as well as other growth regulators such as *MSX2* (Figure 5A). Results of Q-RT-PCR analysis showed the up-regulation of *RET* and *MSX2* in SUM-44 and SUM-52 cells compared with MCF10A cells (Figure 5B and supplemental data). Furthermore, the regulation of *RET* and *MSX2* genes by *SFRP1* was additionally validated in *SFRP1* over expressing SUM-44 cells (Figure 5C). However, no detectable change was found for *CCND1*, *LEF1* and *ANX2* between *SFRP1* over expressing and control breast cancer cells. Thus, *SFRP1* has growth-suppressive activity that may participate in the regulation of the expression of a subset of WNT pathway responsive genes in human breast cancer.

Discussion

Genetic alterations of chromosome arm 8p, such as DNA amplification, loss and chromosomal breaks is one of the most frequent events in a range of common epithelial cancer types including breast, colon, bladder, pancreas and others 4-7. The 8p11-12 region is amplified in approximately 15% of human breast cancers. Recently, our group and several other groups characterized the 8p11-12 amplification in sporadic breast tumors and breast cancer cell lines using high-resolution array CGH to define the region and describe relevant genes that map in this chromosomal location. Conversely, loss of heterozygosity and DNA copy number decrease within 8p11-12 region are also frequently observed in human cancers 37-39. Chin *et al.* demonstrated that reduced copy number in the 8p11-12 region was associated with poor outcome in some breast cancers 8. They hypothesized that poor clinical outcome in breast cancers with 8p11-12 abnormalities is due to increased genome instability/mutagenesis resulting from either up-or down-regulation of genes encoded in this region. Thus, the 8p11-12 region contains both candidate oncogenes and tumor-suppressor genes that may contribute to breast cancer development and progression.

The *SFRP1* gene is located at the 8p11-12 amplified region in breast cancers. An important finding of this study is that promoter methylation of *SFRP1* is a mechanism for negatively regulating this gene with its growth suppressive activity even in the context of gene amplification. Amplified chromosomal regions such as 8p11-12 usually span several megabases, and potentially harbor many genes. However, the number of target genes activated via amplification is usually limited. Janssen *et al.* revealed that *MYEOV* within the

11q13 amplicon is a coamplified gene with *CCND1*, however its activation is inhibited by promoter methylation in a subset of esophageal squamous cell carcinomas 40. In our previous study, we examined the expression level of 53 genes in breast cancer specimens with and without the 8p11-12 amplicon. Extensive analysis of copy number and expression across the entire panel of tumor specimens resulted in a list of 21 genes that met the statistical criteria for target genes because they are over expressed when amplified. Conversely, the expression levels of several genes including *SFRP1*, *INDO* and *STAR* are not upregulated, even though they are located in the 8p11-12 core amplified region 4. In this study, we demonstrate that *SFRP1* is silenced by promoter methylation in breast cancers even when the *SFRP1* gene is amplified. As *SFRP1* gene loss was also frequently observed in human breast cancers, downregulation of *SFRP1* expression can result from both genetic loss and epigenetic inactivation.

The *SFRP1* gene encodes a negative modulator of the WNT signaling pathway. WNT signaling pathways include the canonical or WNT/beta-catenin pathway and non-canonical or beta-catenin-independent pathways. In the canonical pathway, WNT transduces downstream signals by stabilizing beta-catenin and promoting its binding to the TCF family of transcription factors. Similar to findings by Schlang *et al.* and Bafico *et al.* in breast cancer, we found various levels of nuclear beta-catenin present in breast cancer cell lines (Supplemental Data). Among the non-canonical WNT signaling pathways are two intracellular cascades that consist of the WNT/Ca²⁺ pathway and WNT/PCP pathway. The WNT/Ca²⁺ pathway can stimulate the nuclear factor NFAT and other transcription factors like CREB (cAMP Response Element-Binding Protein-1). In the WNT/PCP pathway, WNT proteins bind to Frizzled transmembrane receptors on the cell surface. This causes activation of the Rho/Rac small GTPase and Jun N-terminal Kinase (JNK) via Dsh and subsequently regulates cytoskeletal organization and gene expression. Recent studies demonstrate that WNT proteins activate a complex intracellular signaling network rather than individual pathways 41. WNT-5A is able to activate not only calcium signalling and JNK, but also functions in the beta-catenin pathway. Also, Frizzled receptors have the ability to activate different WNT signaling, *i.e.*, FZD7 can activate canonical as well as non-canonical signaling branches 41. Shih *et al.* demonstrated that *SFRP1* might inhibit WNT signaling through the canonical pathway as well as through catenin-independent non-canonical pathways in hepatocellular carcinoma 42. As we have shown here, stable re-expression of *SFRP1* in SUM-44 and SUM-52 cells resulted in suppressed canonical and non-canonical WNT pathways. *SFRP1* may play a role in regulating WNT signaling networks in the tumorigenesis of human mammary tissues.

Aberrant activation of WNT signaling has been identified in a wide range of human cancers, mostly resulting from mutations in beta-catenin or other WNT pathway components 43. However, such mutations in the WNT pathway have rarely been identified in breast cancer. Nevertheless, there is evidence demonstrating that beta-catenin protein levels are frequently stabilized, and then activated, in breast cancer tumors 44-45. Moreover, constitutive expression of Wnt in mouse mammary epithelial cells induces tumor formation, demonstrating that the hyperactivation of this pathway is a powerful oncogenic signal 46-47. In this study, we demonstrated that *SFRP1* is repressed in a panel of breast cancer cell lines and primary breast cancer specimens. Repression of *SFRP1* may be necessary for the transformed phenotype, including anchorage independent growth, of two breast cancer cell lines (SUM44 and SUM52). Furthermore, we demonstrated that silencing of *SFRP1* resulted in the elevated expression of a subset of WNT/beta-catenin target genes in human breast cancer cells, including *CCND1* and *RET*, which are transforming oncogenes in multiple tumor types. The potent suppressive activity of *SFRP1* in these two cell lines suggests that WNT signaling is required for the transformed phenotypes of these breast cancer cells.

There are 19 WNT genes, 5 SFRP genes and many regulators involved in the WNT pathway in mammals. Several studies describe over expression of individual WNTs as well as down-regulation of other members of the SFRP family in breast cancer [12, 44]. Indeed, our expression profiling data showed that expression of the Dickkopf-1 (DKK1) and Dickkopf-3 (DKK3) was down-regulated in several breast cancer cell lines (Supplemental Data). Furthermore, *C8orf4* (*TC-1*), also located at the 8p11-12 region, encodes a novel protein that interacts with Chibby (Cby), which negatively regulates β -catenin mediated transcription [48-50]. We recently reported that TC-1 over expression is transforming and may link with the FGFR and WNT pathways in a subset of breast cancers. Recent studies suggest that different Wnt signaling pathways are simultaneously active within the same cell type; supporting the idea that Wnt pathways, including canonical and non-canonical, are highly connected to form a Wnt signaling network [18, 19, 41]. The outcome of the beta-catenin-dependent and independent pathways in an individual tumor may be dependent on a multitude of variables ranging from availability of receptors, downstream effectors and inhibitors to external influences coming from the tumor microenvironment and the extracellular matrix. Further study with more comprehensive approaches, including dominant-negative TCF in breast cancer model cells, will be necessary to define which pathway is, or pathways are vital for SFRP1 tumor suppression function.

To our knowledge, this is the first report to show that suppression of *SFRP1* expression in breast cancer cells with an *SFRP1* gene amplification is attributable to *SFRP1* promoter methylation. Furthermore, restoration of SFRP1 expression resulted in inhibited canonical and non-canonical WNT pathways, and suppressed growth of breast cancer cells in which the *SFRP1* gene had been silenced. Thus, down-regulation of SFRP1 can be triggered by epigenetic and/or genetic events and may contribute to the tumorigenesis of human breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004; 10:789–99. [PubMed: 15286780]
2. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet*. 2007; 8:286–98. [PubMed: 17339880]
3. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007; 128:683–92. [PubMed: 17320506]
4. Yang ZQ, Streicher KL, Ray ME, Abrams J, Ethier SP. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. *Cancer research*. 2006; 66:11632–43. [PubMed: 17178857]
5. Gelsi-Boyer V, Orsetti B, Cervera N, Finetti P, Sircoulomb F, Rouge C, Lasorsa L, Letessier A, Ginestier C, Monville F, Esteyries S, Adelaide J, et al. Comprehensive profiling of 8p11-12 amplification in breast cancer. *Mol Cancer Res*. 2005; 3:655–67. [PubMed: 16380503]
6. Garcia MJ, Pole JC, Chin SF, Teschendorff A, Naderi A, Ozdag H, Vias M, Kranjac T, Subkhankulova T, Paish C, Ellis I, Brenton JD, et al. A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes. *Oncogene*. 2005; 24:5235–45. [PubMed: 15897872]
7. Yang ZQ, Albertson D, Ethier SP. Genomic organization of the 8p11-p12 amplicon in three breast cancer cell lines. *Cancer genetics and cytogenetics*. 2004; 155:57–62. [PubMed: 15527903]

8. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo WL, Lapuk A, Neve RM, Qian Z, Ryder T, Chen F, Feiler H, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiology. *Cancer cell*. 2006; 10:529–41. [PubMed: 17157792]
9. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD, Umansky SR. SARPs: a family of secreted apoptosis-related proteins. *Proc Natl Acad Sci U S A*. 1997; 94:13636–41. [PubMed: 9391078]
10. Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *The Journal of biological chemistry*. 1999; 274:16180–7. [PubMed: 10347172]
11. Taketo MM. Shutting down Wnt signal-activated cancer. *Nat Genet*. 2004; 36:320–2. [PubMed: 15054482]
12. Turashvili G, Bouchal J, Burkadze G, Kolar Z. Wnt signaling pathway in mammary gland development and carcinogenesis. *Pathobiology*. 2006; 73:213–23. [PubMed: 17314492]
13. Veeck J, Niederacher D, An H, Klopocki E, Wiesmann F, Betz B, Galm O, Camara O, Durst M, Kristiansen G, Huszka C, Knuchel R, et al. Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis. *Oncogene*. 2006; 25:3479–88. [PubMed: 16449975]
14. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S. Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in human breast cancer. *Cancer Biol Ther*. 2006; 5:281–6. [PubMed: 16410723]
15. Klopocki E, Kristiansen G, Wild PJ, Klamann I, Castanos-Velez E, Singer G, Stohr R, Simon R, Sauter G, Leibiger H, Essers L, Weber B, et al. Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors. *Int J Oncol*. 2004; 25:641–9. [PubMed: 15289865]
16. Zhou Z, Wang J, Han X, Zhou J, Linder S. Up-regulation of human secreted frizzled homolog in apoptosis and its down-regulation in breast tumors. *International journal of cancer*. 1998; 78:95–9.
17. Ugolini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adelaide J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D, Pebusque MJ. WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. *Oncogene*. 2001; 20:5810–7. [PubMed: 11593386]
18. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA. An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer cell*. 2004; 6:497–506. [PubMed: 15542433]
19. Schlange T, Matsuda Y, Lienhard S, Huber A, Hynes NE. Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation. *Breast Cancer Res*. 2007; 9:R63. [PubMed: 17897439]
20. Cowling VH, D'Cruz CM, Chodosh LA, Cole MD. c-Myc transforms human mammary epithelial cells through repression of the Wnt inhibitors DKK1 and SFRP1. *Mol Cell Biol*. 2007; 27:5135–46. [PubMed: 17485441]
21. Forozan F, Veldman R, Ammerman CA, Parsa NZ, Kallioniemi A, Kallioniemi OP, Ethier SP. Molecular cytogenetic analysis of 11 new breast cancer cell lines. *British journal of cancer*. 1999; 81:1328–34. [PubMed: 10604729]
22. Yang ZQ, Imoto I, Fukuda Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano S, Nakamura Y, Inazawa J. Identification of a novel gene, GASC1, within an amplicon at 9p23-24 frequently detected in esophageal cancer cell lines. *Cancer research*. 2000; 60:4735–9. [PubMed: 10987278]
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) Method. *Methods (San Diego, Calif)*. 2001; 25:402–8.
24. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A*. 1996; 93:9821–6. [PubMed: 8790415]
25. Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG. The Wnt antagonist sFRP1 in colorectal tumorigenesis. *Cancer research*. 2004; 64:883–8. [PubMed: 14871816]

26. Moffa AB, Tannheimer SL, Ethier SP. Transforming potential of alternatively spliced variants of fibroblast growth factor receptor 2 in human mammary epithelial cells. *Mol Cancer Res.* 2004; 2:643–52. [PubMed: 15561780]
27. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics.* 2004; 20:307–15. [PubMed: 14960456]
28. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics.* 2005; 21:2067–75. [PubMed: 15657102]
29. Yang C, Ionescu-Tiba V, Burns K, Gadd M, Zukerberg L, Louis DN, Sgroi D, Schmidt EV. The role of the cyclin D1-dependent kinases in ErbB2-mediated breast cancer. *The American journal of pathology.* 2004; 164:1031–8. [PubMed: 14982856]
30. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia.* 2004; 6:1–6. [PubMed: 15068665]
31. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. *Cancer cell.* 2006; 9:121–32. [PubMed: 16473279]
32. Radvanyi L, Singh-Sandhu D, Gallichan S, Lovitt C, Pedyczak A, Mallo G, Gish K, Kwok K, Hanna W, Zubovits J, Armes J, Venter D, et al. The gene associated with trichorhinophalangeal syndrome in humans is overexpressed in breast cancer. *Proc Natl Acad Sci U S A.* 2005; 102:11005–10. [PubMed: 16043716]
33. Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci U S A.* 1999; 96:9212–7. [PubMed: 10430922]
34. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A.* 2001; 98:10869–74. [PubMed: 11553815]
35. Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, Klein J, Fridman E, Skarda J, Srovnal J, Hajduch M, Murray P, et al. Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer.* 2007; 7:55. [PubMed: 17389037]
36. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer cell.* 2006; 10:515–27. [PubMed: 17157791]
37. Dahiya R, Perinchery G, Deng G, Lee C. Multiple sites of loss of heterozygosity on chromosome 8 in human breast cancer has differential correlation with clinical parameters. *Int J Oncol.* 1998; 12:811–6. [PubMed: 9499440]
38. Charafe-Jauffret E, Moulin JF, Ginestier C, Bechlian D, Conte N, Geneix J, Adelaide J, Noguchi T, Hassoun J, Jacquemier J, Birnbaum D. Loss of heterozygosity at microsatellite markers from region p11-21 of chromosome 8 in microdissected breast tumor but not in peritumoral cells. *Int J Oncol.* 2002; 21:989–96. [PubMed: 12370745]
39. Adelaide J, Huang HE, Murati A, Alsop AE, Orsetti B, Mozziconacci MJ, Popovici C, Ginestier C, Letessier A, Basset C, Courtay-Cahen C, Jacquemier J, et al. A recurrent chromosome translocation breakpoint in breast and pancreatic cancer cell lines targets the neuregulin/NRG1 gene. *Genes Chromosomes Cancer.* 2003; 37:333–45. [PubMed: 12800145]
40. Janssen JW, Imoto I, Inoue J, Shimada Y, Ueda M, Imamura M, Bartram CR, Inazawa J. MYEOV, a gene at 11q13, is coamplified with CCND1, but epigenetically inactivated in a subset of esophageal squamous cell carcinomas. *J Hum Genet.* 2002; 47:460–4. [PubMed: 12202983]
41. Kestler HA, Kuhl M. From individual Wnt pathways towards a Wnt signalling network. *Philos Trans R Soc Lond B Biol Sci.* 2008; 363:1333–47. [PubMed: 18192173]
42. Shih YL, Hsieh CB, Lai HC, Yan MD, Hsieh TY, Chao YC, Lin YW. SFRP1 suppressed hepatoma cells growth through Wnt canonical signaling pathway. *International journal of cancer.* 2007; 121:1028–35.

43. Behrens J, Lustig B. The Wnt connection to tumorigenesis. *Int J Dev Biol.* 2004; 48:477–87. [PubMed: 15349822]
44. Mohinta S, Wu H, Chaurasia P, Watabe K. Wnt pathway and breast cancer. *Front Biosci.* 2007; 12:4020–33. [PubMed: 17485355]
45. Brennan KR, Brown AM. Wnt proteins in mammary development and cancer. *J Mammary Gland Biol Neoplasia.* 2004; 9:119–31. [PubMed: 15300008]
46. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell.* 1988; 55:619–25. [PubMed: 3180222]
47. Kwan H, Pecanka V, Tsukamoto A, Parslow TG, Guzman R, Lin TP, Muller WJ, Lee FS, Leder P, Varmus HE. Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. *Mol Cell Biol.* 1992; 12:147–54. [PubMed: 1530875]
48. Jung Y, Bang S, Choi K, Kim E, Kim Y, Kim J, Park J, Koo H, Moon RT, Song K, Lee I. TC1 (C8orf4) enhances the Wnt/beta-catenin pathway by relieving antagonistic activity of Chibby. *Cancer Res.* 2006; 66:723–8. [PubMed: 16424001]
49. Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, Moon RT. Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. *Nature.* 2003; 422:905–9. [PubMed: 12712206]
50. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem.* 2006; 281:22429–33. [PubMed: 16793760]

Figure 1.

(A) Cartoon of the 8p11-12 amplified region in 2 breast cancer cell lines and 3 primary samples. Localization of the 8p11-12 genes including *SFRP1* are represented to the right of the chromosome 8 ideogram. The lines at far right represent the amplified region of each sample based on the array-CGH data. (B) Down-regulation of *SFRP1* in primary breast cancer specimens. *SFRP1* mRNA levels were analyzed by using gene expression data sets from the cancer gene microarray meta-analysis public database, Oncomine. The Y-axis represents normalized expression units. Shaded boxes represent the interquartile range between the 25th and 75th percentile; whiskers represent the 10th to 90th percentile range; horizontal lines represent the median. The P-value was calculated using Student's *t*-test. The following analyses were performed: (1) normal breast vs. breast carcinoma, $P = 1.1\text{E-}8$ (Richardson *et al.* 31); (2) normal breast vs. breast carcinoma, $P = 1.8\text{E-}5$ (Radvanyi *et al.* 32); (3) normal breast vs. breast ductal carcinoma, $P = 2.7\text{E-}5$ (Perou *et al.* 33); (4) normal breast vs. breast lobular carcinoma, $P = 0.001$ (Perou *et al.* 33); (5) benign breast vs. breast carcinoma, $P = 0.007$ (Sorlie *et al.* 34); (6) adjacent normal ductal vs. invasive ductal carcinoma, $P = 0.015$ (Turashvili *et al.* 35); (7) adjacent normal lobular vs. invasive lobular carcinoma, $P = 0.04$ (Turashvili *et al.* 35).

Figure 2.

Methylation analysis of *SFRP1* in breast cancer cells. Methylation-specific PCR was performed on bisulphite-treated DNA from (A) 10 SUM breast cancer cell lines and (B) 9 primary breast cancer samples. DNA bands in lanes labeled M indicate PCR products amplified with primers recognizing the methylated promoter sequence. DNA bands in lanes labeled U represent amplified products with an unmethylated-specific primer. The immortalized, nontransformed mammary epithelial cell line, MCF10A, served as the positive control for the U reaction. Water was used instead of template in the negative control (DDW). The stars indicate samples that had *SFRP1* gene amplification and arrows indicate samples that had methylated DNA molecules. (C) Re-expression of *SFRP1* by 5-aza-dC treatment was analyzed by Q-RT-PCR. SUM-44 and SUM-52, and the control cell line MCF10A were treated with 1 μ M 5-aza-dC (AZA) or DMSO for 3 days. *SFRP1* expression level (fold change) in AZA-treated cells relative to DMSO-treated control cells were measured by Q-RT-PCR and normalized to GAPDH expression. For DMSO-treated control cells, the baseline was set to one.

Figure 3.

(A) Expression of *SFRP1* mRNA in SUM-44 and SUM-52 cells after being transfected with the control vector or a SFRP1 construct. SUM-44 and SUM-52 cells were transfected with 2 μ g/well of the pcDNA-GW-SFRP1 expression vector (pc-SFRP1) or pcDNA-GW-CAT control vector using FuGENE HD transfection reagent and cultured in 400 μ g/ml (SUM-44) or 200 μ g/ml (SUM-52) G418 for 4 weeks. *SFRP1* mRNA expression levels were measured by RT-PCR and normalized to GAPDH expression. PCR product from 25 cycles are shown. (B) Colony formation assays of the SUM-44 and SUM-52 cells transfected with the SFRP1 expression vector. The graphs indicate the number of colonies in the SUM-44 and SUM-52 cells transfected with the pcDNA-GW-SFRP1 expression vector (pc-SFRP1) relative to the number of colonies formed after control vector transfection, which was set to 100%. (C) Representative images of colonies in SUM-52 cells are shown. (D) SUM-44 and SUM-52 cell lines were infected with a control lentivirus (pLenti6-LacZ) and a lentivirus encoding SFRP1 (pL-SFRP1=pLenti6-SFRP1) to generate the cell lines indicated. Exogenous expression of SFRP1 in SUM-44-pL-SFRP1 and SUM-52-pL-SFRP1 cells was detected by western blot analysis with anti-V5 antibody. (E) Ectopic SFRP1 expression inhibited anchorage-independent growth *in vitro*. Ectopic SFRP1 expression in SUM-44 and SUM-52 breast cancer cells with the pLenti-SFRP1 lentivirus (pL-SFRP1) reduced colony formation in soft agar compared with cells transduced with the control vector virus. The graph indicates the number of colonies formed by exogenous SFRP1 over expressing cells relative to the number of colonies formed by control cells in soft agar, which was set to 100%.

Figure 4.

Luciferase reporter gene assays were done to compare the activities of TCF, NFAT and AP1 transcription factors in A) SUM-44-SFRP1 and B) SUM-52-SFRP1, which stably express SFRP1. All of the luciferase reporter constructs were identical, including a minimal promoter, except that each contained tandem repeats of a response element for TCF/LEF, NFAT, or AP1. Resulting luciferase activities (in light units) were normalized to protein per assay and shown as a percentage relative to the corresponding lacZ expressing control. Error bars are the standard deviation of each representative assay in triplicate. A similar transfection efficiency and minimal promoter activity for the cell lines were observed by transfection of a GFP expressing vector driven by a minimal promoter (data not shown).

Figure 5.

(A) Unsupervised hierarchical clustering of *SFRP1* and 4 representative Wnt/ β -catenin target genes in 153 breast cancer specimens. Affymetrix-based gene expression profiles of 130 primary breast cancer samples were obtained from published data 8. Each row represents a probe, and each column represents a sample. Green indicates low expression level of the transcript; black indicates intermediate and red indicates high. (B) Up-regulation of *RET* and *MSX2* in SUM-44 and SUM-52 breast cancer cell lines. The mRNA expression level of *RET* and *MSX2* in SUM-44 and SUM-52 breast cancer cells and control MCF10A cells were measured by Q-RT-PCR and normalized to GAPDH expression. The number of cycles to threshold values for *RET* and *MSX2* in SUM-44 and SUM-52 were normalized to values for GAPDH and then compared to *RET* and *MSX2* expression in MCF10A cells to determine their fold change. (C) Down-regulation of *RET* and *MSX2* in SFRP1 over-expressing SUM-44 breast cancer cells. The mRNA expression levels of *RET* and *MSX2* in SFRP1 over-expressing SUM-44 cancer cells and control SUM44-LacZ cells were measured by Q-RT-PCR and normalized to GAPDH expression.

SFRP1 copy number and expression levels for repressive breast cancer cell lines and primary tumors

Table 1

Cell Lines	SUM-44	SUM-52	SUM-102	SUM-149	SUM-185	SUM-190	SUM-225	SUM-229	
aCGH	2.11	4.45	N/A	1.94	1.46	1.62	-2.28	1.16	
Affymetrix	-7.39	-7.77	-2.88	1.37	-6.27	-5.78	-6.14	-7.71	
Tumors	10173	4473	6617	6716	69	3920	3971	7411	4687
aCGH	3.12	1.07	5.96	1.35	2.90	1.38	1.01	N/A	1.15
qRT-PCR	-20.62	-121.25	-44.16	-285.09	-121.43	-65.56	-546.81	-406.44	-38.88

Note: The expression of *SFRP1* from the Affymetrix array is obtained by the average of three probes (See Supplement Data)