

Title: Epigenetic regulation of the secreted frizzled-related protein family in human glioblastoma multiforme

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Abstract

Glioblastoma multiforme (GBM) are intracranial tumors of the central nervous system and the most lethal amongst solid tumors. Current therapy is palliative and is limited to surgical resection followed by radiation therapy and temozolomide treatment. Aberrant WNT pathway activation mediates not only cancer cell proliferation but promotes radiation and chemotherapeutic resistance. WNT antagonists such as the secreted frizzled-related protein (sFRP) family have an ability to sensitize glioma cells to chemotherapeutics, decrease proliferation rate, and induce apoptosis. During tumor development, sFRP genes (1 to 5) are frequently hypermethylated, causing transcriptional silencing. We investigated a possible involvement of methylation-mediated silencing of the sFRP gene family in human GBM using four human glioblastoma cell lines (U87, U138, A172, and LN18). To induce demethylation of the DNA, we inhibited DNA methyltransferases through treatment with 5-Azacytidine. Genomic DNA, RNA, and total protein were isolated from GBM cells before and after treatment. We utilized bisulfite modification of genomic DNA to examine the methylation status of the respective sFRP promoter regions. Pharmacological demethylation of the GBM cell lines demonstrated a loss of methylation in sFRP promoter regions, as well as an increase in sFRP gene-specific mRNA abundance. Western Blot analysis demonstrated an increased protein expression of sFRP-4 and increased levels of phosphorylated- β -catenin. These data indicate an important role of methylation-induced gene silencing of the sFRP gene family in human GBM.

Keywords: epigenetics, gene, glioblastoma, methylation, Wnt signaling

Introduction

Glioblastoma multiforme (GBM) are the most aggressive amongst solid tumors within the central nervous system, arising from glia cells or their precursors¹. Current therapeutic approaches are limited to surgical resection followed by radiation therapy and temozolomide treatment². However, these therapies have little effect on the recurrence rate of these highly invasive brain tumors and their associated poor prognosis. Patients' median survival rate post diagnosis is generally less than one year³. Tumors are characterized through the presence of neoplastic cells, which differ from normal tissue in respect to their proliferation pattern, morphology, and gene expression⁴.

The WNT pathway plays a key role in cell proliferation and consists of highly conserved WNT protein ligands that bind to the frizzled (Fz) receptors. Activation triggers a signal transduction cascade in which glycogen synthase kinase3 (GSK3) and β -catenin are important downstream mediators^{5,6}.

In the absence of WNT signaling, GSK3 phosphorylates cytoplasmic β -catenin, making it a target for degradation by the proteasome⁷. However, during aberrant WNT-pathway signaling activation, GSK3 is inactivated, resulting in increased levels of unphosphorylated cytoplasmic β -catenin. This accumulation of cytoplasmic β -catenin increases its translocation to the nucleus where it activates a variety of target genes involved in tumor development and progression⁸. Therefore, a finely tuned homeostatic balance between the cytoplasmic and nuclear β -catenin determines the final outcome of the WNT signaling pathway. Such balance is brought about through extracellular WNT antagonists such as the secreted frizzled related protein (sFRP) family, which possesses a domain similar to one in the Fz receptor protein(s)⁹. By binding to the Fz receptor, the accumulation of free β -catenin can act as an antagonist, which in turn, counteracts WNT signaling. The sFRP family has shown an ability to sensitize glioma cells to chemotherapeutics, decrease their proliferation rate, and induce apoptosis¹⁰. It

has been shown in various tumors, particularly during tumor development, that these genes are frequently hypermethylated, and thereby inducing transcriptional silencing¹¹.

Downregulation and gene silencing of the sFRP family through epigenetic modifications have been demonstrated in colorectal and prostate cancers¹². DNA methylation has emerged as an additional and important mechanism in the process of epigenetic gene silencing¹³. The objective of this study was to investigate and examine the involvement of methylation-mediated silencing of the sFRP gene family in human GBM, using four different GBM cell lines. In order to induce demethylation of the DNA, we inhibited DNA methyltransferases through treatment with 5-Azacytidine (5-Aza), a cytidine analogue that incorporates into the chemical structure of DNA and RNA.

Materials and Methods

Cell culture

A total of four human glioblastoma (GBM) cell lines (U87, U138, A172, and LN18) were obtained from American Type Culture Collection (Manassas, USA). U87 and LN18 are able to form solid tumors after inoculation in nude mice, whereas U138 and A172 show no tumorigenicity (www.atcc.org). U87, U138, and A172 cells were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (*HyClone*, Logan, USA) and 1% penicillin streptomycin sulfate (*HyClone*, Logan, USA) at 5% CO₂, 37°C, and 95% humidity. LN18 Cells were cultured in DMEM-F12 medium containing 5% fetal bovine serum and 1% penicillin streptomycin sulfate at 5% CO₂, 37°C, and 95% humidity. To analyze the effects of 5-Azacytidine, cells were cultured in media supplemented with the compound.

Treatment

5-Azacytidine (5-Aza) was purchased from Sigma-Aldrich (St. Louis, USA). After establishing a dose response effect based on results from a MTT Viability assay, cells were incubated with 20 μ M 5-Aza for 72h after a 24 h seeding period since this dose and treatment time provided consistent reduction in cell viability across all cell lines. Media containing the drug were changed every day.

Viability Assay

GBM cells were seeded into 96-well plates at a density of 2×10^4 per well (100 μ l). Wells with drug-free medium were used as negative control. The cells were treated for 24h, 48h, or 72h. At 4h before the desired time points, 10 μ l of MTT solution (5 mg/ml) was added into each well and cells were incubated at 5% CO₂, 37°C, and 95% humidity for another 4h. The medium was removed and 100 μ l of DMSO were added into each well. The absorbance was detected at 570 nm with a Microplate Reader.

DNA Isolation

Genomic DNA was extracted from all cell lines using a GeneJET Genomic DNA Purification Kit (Fermentas, Thermo Fisher Scientific Inc., Canada) according to the manufacturer's instructions. DNA integrity and quality were evaluated by spectrophotometric analysis.

RNA extraction and reverse transcription

Total RNA was extracted from human GBM cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The RNA pellet was dissolved in 50 μ L of DEPC treated water. RNA integrity and quality were evaluated through both gel electrophoresis and spectrophotometric analysis.

The RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions, using 1 µg of total RNA as input material.

PCR

PCR reactions were employed using 10 µl of 2x PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µM of each forward and reverse primer, 1 µl of template cDNA, and water adjusted to 20 µl. PCR products were analyzed by agarose gel electrophoresis. Primers¹⁴ used for PCR amplification of this cDNA are given in Table 1.

Table 1 qPCR Primer Sequences and annealing temperatures

Methylation-specific PCR

The methylation status of all 5 sFRP genes in human GBM cell lines was determined by methylation specific polymerase chain reaction (MSP). Briefly, 1 µg of genomic DNA was bisulfite-treated with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite-treated DNA was amplified using 10 µl of 2x PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µM of each forward and reverse primer, 1 µl of template DNA, and water adjusted to a final volume of 20 µl. PCR products were analyzed by agarose gel electrophoresis. Previously described primers specific for either methylated or unmethylated DNA¹² are shown in Table 2.

Table 2 MSP Primer Sequences and annealing temperatures

Real-time PCR

Quantitative real-time (qPCR) was performed using an AB ViiA 7 real-time PCR system (Applied Biosystems, Foster City, CA) and KAPA qPCR SYBR green PCR Master Mix (Geneworks, Australia). Reaction mixtures consisted of 5 µl of 2× KAPA SYBR green

Master Mix, 0.5 μ M of each forward and reverse primer, 1 μ l of template, 0.2 μ M ROX low, and water adjusted to 10 μ l. Each reaction mixture was loaded into 96-well PCR plates and amplified under PCR cycling conditions described in Table 2, followed by a melt-curve analysis.

Western blots

Protein was extracted from cells using radioimmunoprecipitation (RIPA) buffer. Protein Detection and Quantitation was estimated using the Direct Detect[®] Spectrometer (Millipore, Temecula, CA). Electrophoretic separation of proteins was performed by discontinuous (Laemmli) SDS-PAGE using the Mini-PROTEAN[®] II electrophoresis cell (Bio-Rad). Thereafter, proteins were transferred onto nitrocellulose membranes using the iBlot gel transfer device (Invitrogen). Membranes were then incubated for 1h in blocking solution (20 mM Tris pH 7.6, 140 mM NaCl, 0.1% Tween-20, 5% w/v non-fat dry milk) and then washed three times for 5 min with Tris-buffered saline containing Tween-20 (TBS-T: 20 mM Tris pH 7.6, 140 mM NaCl, 0.01% Tween-20). Primary antibodies (sFRP-4, β -Actin, phosphorylated- β -catenin, and total β -catenin) were diluted 1:1000 with PBS-T containing 5% w/v BSA, and the membranes were incubated overnight at 4°C with gentle agitation. Secondary antibodies (Anti-Rabbit-IgG) were diluted 1:2000 with PBS-T containing 5% w/v BSA, and the membranes were incubated for 1h at 4°C with gentle agitation.

Statistics

Statistical analysis was performed with GraphPad Prism V5.01 (GraphPad Software, La Jolla, USA) using a two-tailed Student's t-test showing significance with $P < 0.05$ as *; $P < 0.01$ as **; and $P < 0.001$ as ***. Data are presented as mean \pm standard deviation (SD).

Results

Effect of 5-Aza on GBM cells

Tumor cell viability was determined in the presence of increasing drug (5-Aza) concentrations using a total of 4 GBM cell lines. The 5-Aza concentrations ranged from 0 μ M in the untreated control to 30 μ M, while the incubation period was 24h, 48h, and 72h (Figure 1) Viability of the treated cells is reported relative to their untreated control.

Figure 1 MTT dose response results for 4 GBM cell lines (U87, U138, LN18, and A172) treated with increasing concentrations of 5-Azacytidine for 24h, 48h, and 72h (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

There was no significant decrease in cell viability in U87 after 24h treatment with any concentration of 5-Aza. However, viability decreased significantly to about 50% after 48h of treatment, even in samples treated with the lowest dose. After incubation for 72h, only the cells treated with 30 μ M 5-Aza showed an additional decrease in viability to less than 50%.

The treatment of U138 cells with 5-Aza had no significant effect on the viability of the cells after 24h incubation. Measurements after 48h of treatment indicated a response to rising concentrations of the drug. A reduction in viability of about 20% compared to the control was measured in the cells treated with 30 μ M 5-Aza for 48h. U138 showed a similar response to U87 after the 72h treatment period. Cell viability decreased $>50\%$ in samples treated with 20 μ M and 30 μ M 5-Aza.

In LN18 cells, no significant change in viability was measured after 24h and 48h treatment, apart from samples that were treated with 30 μ M for 48h. 72h after incubation, more significant results were seen, as the viability decreased rapidly to about 50% compared to the control.

Cell line A172 showed a similar pattern in every treatment group. Viability decreased more in cells treated with higher concentrations of the drug. However, no IC₅₀ value was achieved, even after 72h of treatment with 30μM of 5-Aza.

Methylation analysis of the sFRP promoters in human GBM

The chosen 5-Aza treatment (20μM for 72h) was performed on the four GBM cell lines to introduce demethylation into their genomic DNA. Isolated DNA was then bisulfite treated for use in the MSP experiments. MSP is a sensitive technique designed to investigate changes in DNA methylation of CpG dinucleotides within promoter regions, so called CpG islands. We used MSP to detect methylation patterns in promoters of the five human sFRP genes in human GBM cells to distinguish between methylated and unmethylated DNA. Methylation analysis was performed pre- and post-treatment with 5-Aza. Figure 2 shows the results of the MSP performed on DNA of all cell lines and for all genes investigated.

Figure 2 MSP results for sFRP-1,2,3,4, and 5 analyzed in U87, U138, LN18, and A172 cells.

All four GBM cell lines showed a hypermethylated (M) promoter region of all sFRPs in the untreated cells. However, following treatment, all cell lines lacked promoter methylation in the analyzed region, as indicated by exclusive amplification with primers specific for the unmethylated DNA sequence.

In vitro demethylation of the sFRP promoters

To prove a direct association of sFRP promoter demethylation with an increased sFRP-specific mRNA expression, we treated all four GBM cell lines with 20μM of 5-Aza. We determined sFRP promoter methylation and sFRP expression before and after the drug treatment. mRNA analyses after drug treatment (Figure 3) confirmed that sFRP promoter

demethylation had occurred in all of the originally methylated cell lines by the presence of signals indicating an unmethylated sFRP promoter. Those cell lines gaining unmethylated promoter sequence after treatment consistently showed elevated sFRP mRNA expression.

Figure 3 Real-time qPCR results for sFRP-1,2,3,4,5 and ABCG2 analyzed in U87, U138, LN18, and A172 cells.

sFRP-1 mRNA induction, as determined by real-time PCR, was up to 15-fold (U87) compared to originally methylated cells. However, sFRP-1 mRNA expression in A172 did not undergo significant changes (Figure 3, sFRP-1, D).

Figure 3 compares sFRP-2 mRNA expression between untreated and 5-Aza treated samples, demonstrating a significant upregulation in samples with a demethylated promoter region. The fold change of sFRP-2 upregulation with respect to the matching control was up to 3-fold as shown in U138 (Figure 3, sFRP-2, B). A distinct increase of sFRP-2 mRNA expression could be observed in 2 of the 4 cell lines, with no change in LN18 and no detectable expression in A172 cells.

Demethylation of its promoter had a functional effect on expression of sFRP-3 specific mRNA. Upon treatment with 5-Aza, expression of sFRP-3 mRNA increased up to 3-fold (Figure 3, sFRP-3, A) in all cell lines but LN18, where the expression levels were not significantly different to its untreated control.

The sFRP-4 mRNA expression levels were significantly elevated in all cell lines and ranged from 1.6-fold (Figure 3, sFRP-4, C) to 3.5-fold (Figure 3, sFRP-4, B).

Results for the sFRP-5 expression demonstrate a rise in mRNA levels of up to 5-fold throughout the four cell lines post-treatment with 5-Aza.

To include a positive control to the mRNA analysis, we investigated the expression levels of the ABCG2 drug transporter, where 5-Aza treatment has been shown to lead to increased mRNA expression in renal carcinoma¹⁵.

Western blotting

After

investigating the methylation patterns of the sFRP promoter regions pre- and post-treatment with the DNA demethylating drug 5-Aza, showing a loss of methylation in all genes and increased mRNA expression after treatment, we addressed the effect on the protein expression.

Figure 4 Western Blot results for sFRP-4, phosphorylated- β -catenin, total β -catenin, and β -actin analyzed in U87, U138, LN18, and A172 cells.

Figure 4 shows the results of Western blotting using rabbit anti-human sFRP-4, phosphorylated- β -catenin, total β -catenin, and β -actin polyclonal antibodies.

sFRP-4 protein expression increased up to 2.3-fold in A172 cells after treatment. Phosphorylated β -catenin protein levels showed a significant elevation of up to 50% compared to the control, while the amount of total- β -catenin and β -actin remained unaltered by the treatment with 5-Aza.

Discussion

Tumor cells commonly alter their protein expression profile in response to increased or decreased modifications in the methylation status of specific genes¹⁶. Changes in DNA methylation have been proposed to play an important role in the aberrant expression of tumor cell genes¹⁷. Experimental data obtained using cultured cells that were treated with 5-Azacytidine, which causes a stable and conserved DNA hypomethylation¹⁸ and activation of

enzyme genes such as O6-methylguanine-DNA methyltransferase (MGMT), substantiate this hypothesis.

MGMT is a DNA repair enzyme that removes mutagenic and cytotoxic adducts from the O6 position of guanine, but it is usually downregulated during tumorigenesis¹⁹. In tumor tissue, activation of the MGMT pathway through demethylation of its promoter has been shown to induce cell death²⁰.

Altered gene expression due to epigenetic silencing processes such as DNA methylation of their gene promoter, has been previously reported²¹. Typically, the altered genes include those involved in important pathways such as proliferation, apoptosis, and WNT signalling²². The WNT pathway is essential in many biological processes such as proliferation, differentiation, migration and survival of cells²³⁻²⁶ but its aberrant activation plays a key role in tumorigenesis⁸.

Several new pathway contributors have been discovered over recent years and a conclusion has been formed that the involvement of canonical WNT signaling in the manifestation of cancer is most likely due to inappropriate gene activation mediated through β -catenin²⁷.

Canonical WNT signaling can be inhibited by a variety of antagonists, such as the sFRP gene family, which is often regulated and silenced due to promoter hypermethylation in human carcinogenesis^{12,28,29}. Of the 5 currently known sFRP isoforms, *sFRP-1* and *sFRP-2* have been identified as targets of epigenetic inactivation in human brain cancers such as astrocytic gliomas to date³⁰, and we asked whether additional members of the sFRP family might be affected by epigenetic silencing in human glioblastoma multiforme.

Hypermethylation of sFRP-1, sFRP-2, sFRP-3, sFRP-4, and sFRP-5 was detected in all four GBM cell lines (U87, U138, LN18, A172) examined before pharmacological demethylation of their genomic DNA through incubation with 20 μ M 5-Azacytidine for 72h. Reports on the involvement of hypermethylation in regulation of the sFRP gene family have been previously

reported dealing with mesothelioma, colorectal cancer, and gastric cancer^{12,28,31,32} and substantiate our findings in GBM. sFRP-1 protein expression was studied in renal cell carcinoma³³. sFRP-1 promoters were shown to be hypermethylated in renal cell carcinoma specimens and showed a significant downregulation of their mRNA levels compared to normal kidney tissue. Hypermethylation of the promoter regions correlated with almost complete loss of sFRP-1 at the protein level, indicating involvement of methylation mediated silencing³³ and substantiates our findings.

Re-expression of sFRP-2 in mammary cells has been shown to substantially inhibit proliferation rates after treatment³⁴. This was assessed through detection of the growth promoting gene *cyclin D1*, which is a direct indicator for active Wnt signaling³⁵. Promoter hypermethylation of sFRP-2 has been previously shown in human gastric cancer³², which agrees with our findings in all GBM cell lines pre-treatment.

However, the involvement of DNA methylation seems unclear in studies focusing on sFRP-3. Suzuki et al.¹² claim that the sFRP-3 promoter does not possess CpG-Islands in its promoter and is therefore not affected by DNA methylation, and processes involved with downregulation of this gene are still unclear.

However, others detected methylation-dependent downregulation of sFRP-3 mRNA in a methylation analysis of renal cell carcinoma³⁶ and melanoma³⁷. Furthermore, Lin *et al.* have detected methylation of sFRP-3 in hepatocellular carcinoma³⁸ and Marsit et al. described increased methylation of the sFRP-3 promoter in bladder cancer²⁹. These previous reports substantiate our findings on methylated sFRP-3 promoter sequences and, therefore, we suggest that gene methylation is a major reason for the down-regulation of sFRP-3 in human GBM.

Moreover, we showed a loss of methylation within promoter regions after treatment and bisulfite conversion, using methylation specific PCR and primers specific for unmethylated

promoter regions of each sFRP gene. Therefore, our results suggest that epigenetic processes mediated through methylation of 5'-CpG-islands in the promoters of the sFRP gene family are involved and may be a common alteration in human glioblastoma multiforme cell lines.

In addition, the investigations of sFRP-specific mRNA abundance before and after treatment revealed a significant increase of sFRP-1 (3 out of 4 cell lines), sFRP-2 (2 out of 3 cell lines), sFRP-3 (3 out of 4 cell lines), sFRP-4 (4 out of 4 cell lines) and sFRP-5 (4 out of 4 cell lines) expression after DNA-demethylating treatment, demonstrating that the sFRPs are transcriptionally silenced by epigenetic processes in human glioblastoma multiforme. This has been shown by Qi et al. in colorectal tumor³⁹, where silenced sFRP genes were associated with promoter hypermethylation, and reactivated sFRP mRNA expression in colorectal cancer through demethylating treatment. Subsequent studies further demonstrated that the overexpression of sFRP-1, sFRP-2, sFRP-4, and sFRP-5 downregulated WNT signaling in colorectal cancer cells¹², where the lack of basal mRNA expression was restored by a treatment with demethylating 5'-aza-2'-deoxycytidine. mRNA expression after demethylating treatment was also studied in esophageal adenocarcinoma, which resulted in re-expressed sFRP mRNA in esophageal adenocarcinoma cancer cell lines⁴⁰.

Our Western blotting analysis demonstrated increased sFRP-4 and phosphorylated β -catenin expression after demethylating treatment. Zou et al. demonstrated that the sFRP-4 protein expression was downregulated in samples with hypermethylated promoter regions, while studies conducted in human mesothelioma cell lines state that sFRP-4 is silenced by promoter hypermethylation³¹. These studies confirm our hypothesis that methylation appears to functionally silence sFRP-4 gene expression in human GBM.

In leukemia, the lack of sFRP-5 protein expression was correlated with the hypermethylation of its promoter. Expression could be restored using a DNA demethylating agent⁴¹. Restoration of the expression of sFRP-5 through demethylation, attenuated Wnt signaling in

ovarian cancer cells and suppressed cancer cell growth, invasion of cells, and tumorigenicity in mice⁴².

Another group stated that treatment of the myeloid leukemia cell lines treated with demethylating agent 5'-aza-2'-deoxycytidine induced re-expression of previously methylated sFRP-1, sFRP-2, sFRP-4, and sFRP-5, and resulted in inactivation of the Wnt pathway by downregulating important Wnt pathway genes, and reducing nuclear localization of active, unphosphorylated β -catenin protein⁴³. This corroborates well with our observations in this study demonstrating an increased expression of inactive phospho β -catenin protein upon 5-Aza treatment.

A loss of sFRP expression through epigenetic silencing may affect cell proliferation via activation of the WNT pathway, thereby potentially enhancing tumor growth and promoting malignant transformation and cancer cell survival. Demethylation of the sFRP promoter regions and the increased expression of their corresponding proteins may lead to inhibition of aberrantly activated cellular networks, thus attenuating tumorigenesis.

In summary, our data suggest that epigenetic silencing of the sFRPs may be a key feature in human glioblastoma multiforme and is most likely due to hypermethylation of its promoter. Taking into account their ability to counter cell growth and to introduce apoptosis, the epigenetic status of the sFRP gene family might serve as a potent therapeutic marker for this highly aggressive brain tumor.

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Conflict of interest

The authors have no conflict of interest.

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Figure Legends

Figure 1. MTT dose response results for 4 GBM cell lines (U87, U138, LN18, and A172) treated with increasing concentrations of 5-Azacytidine for 24h, 48h, and 72h (* P = 0.05, ** P = 0.01, *** P = 0.001).

Figure 2. MSP results for sFRP-1, 2, 3, and 5 analyzed in U87, U138, LN18, and A172 cells. M: Primer specific for methylated DNA. U: Primer specific for unmethylated DNA. Control: untreated cells. 5-Aza: 20 μ M 5-Azacytidine for 72h.

Figure 3. Real-time qPCR results for sFRP-1,2,3,4,5 and ABCG2 analyzed in U87, U138, LN18, and A172 cells. White bars: Untreated controls; Grey bars: Relative mRNA expression of 20 μ M 5-Azacytidine for 72h treated cells to their untreated control (*= p< 0.05, **= p<0.01, ***= p< 0.001).

Figure 4. Western Blot results for sFRP-4, phosphorylated β -catenin, total β -catenin, and β -actin analyzed in U87, U138, LN18, and A172 cells. White bars: Untreated controls; Grey bars: Relative protein expression of 20 μ M 5-Azacytidin for 72h treated cells to their untreated control, quantified via densitometric analysis (*= p< 0.05, **= p<0.01, ***= p< 0.001).

Table 2. qPCR Primer Sequences and annealing temperatures

Gene	Forward(5'-3')	Reverse(5'-3')	qPCR AT (°C)
sFRP-1	atctctgtgccagcgagttt	aagtggaggctgaggtgtc	65
sFRP-2	aggacaacgaccttgcac	ttgctctggctccaggat	65
sFRP-3	aaactgtagaggggcaagca	ggcagccagagctggtatag	65
sFRP-4	cgatcgggtcaagtgtaaa	gacttgagttcgaggatgg	65
sFRP-5	gatgtgctccagtgactttg	gcaggggtaggagaacatga	65

Table 2. MSP Primer Sequences and annealing temperatures

Gene	Specificity	Forward(5'-3')	Reverse(5'-3')	AT (°C)
sFRP-1	Methylated	gtgtcgcgcttcgctgttcgc	aacgttaccgactccgcgaccg	60
	Unmethylated	gagtagtggtgtgtttgtttgt	cccaacattaccaactccacaacca	60
sFRP-2	Methylated	gggtcggagttttcggagtcgcg	ccgctctcttcgctaaatacgactcg	62
	Unmethylated	tttgggttggagtttttggagttgt	aaccactctcttcactaaatacaactca	66
sFRP-3	Methylated	ggagcgggtttttggcg	gaaccccgaaacaccgaaa	65
	Unmethylated	ggagtgggtttttggtgtttattgt	cccaaaccctaacacca	63
sFRP-4	Methylated	gggtgatgttatcgttttgcgac	cctcccctaacgtaaacgaaacg	63
	Unmethylated	gggggtgatgtattgttttgcgac	cacctcccctaacataaacctcaaaaca	60
sFRP-5	Methylated	aagatttggcgttggcgggacgttc	actccaaccgaaacctcgccgtacg	65
	Unmethylated	gtaagatttgggttgggtgggatgtt	aaaactccaaccctaacctcaccataca	68







