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***SFRP1* promoter methylation and expression in human trabecular meshwork cells**

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Abstract

Glaucoma is a leading cause of blindness worldwide. In primary open angle glaucoma (POAG) patients, impaired trabecular meshwork (TM) function results in elevated intraocular pressure (IOP), which is the primary risk factor of developing optic neuropathy. Our previous studies showed that Wnt signaling pathway components are expressed in the human TM (HTM), and the Wnt inhibitor, *secreted frizzled-related protein 1* (*SFRP1*) is elevated in the glaucomatous TM (GTM). Elevated *SFRP1* increased IOP in mice eyes and in perfusion cultured anterior segments of the human eye. However, the cause of elevated *SFRP1* in the GTM remains unknown. Promoter methylation plays a key role in regulating *SFRP1* expression in certain cancer cells. In light of this, we studied whether promoter methylation is also involved in *SFRP1* differential expression in the TM. Two normal TM (NTM) and two GTM cell strains were cultured for an additional 7 days after they were confluent. RNA and genomic DNA (gDNA) were isolated simultaneously to compare *SFRP1* expression levels by quantitative PCR (qPCR) and to determine *SFRP1* promoter methylation status by bisulfite conversion and methylation-sensitive high resolution melting analysis (MS-HRM). To study whether DNA methylation inhibitors affect *SFRP1* expression in TM cells, the four TM cell strains were treated with or without 2 μ M 5-aza-2'-deoxycytidine (AZA-dC) for 4 days. RNA was isolated to compare *SFRP1* expression by qPCR. In addition, a human cancer cell line, NCI-H460, was used as a positive control. We found that the two GTM cell strains had significantly higher expression levels of *SFRP1* than the two NTM cell strains. However, the *SFRP1* promoter of all four TM cell strains was unmethylated. In addition, AZA-dC treatment did not affect *SFRP1* expression in any of the TM cell strains ($n = 3$, $p > 0.05$). In contrast, the hypermethylated *SFRP1* promoter of NCI-H460 cells was partially demethylated by the same treatment. AZA-dC treatment also elevated *SFRP1* expression by approximately two fold in NCI-H460 cells ($n = 3$, $p < 0.01$). Our data suggest that the differential expression of *SFRP1* in HTM cells is not due to differential promoter methylation.

Keywords

glaucoma; intraocular pressure; trabecular meshwork; *SFRP1*; DNA methylation; 5-aza-2'-deoxycytidine

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1. Introduction

Glaucoma is a leading vision-threatening disease worldwide. Although the mechanism of this ocular disease is not clear, numerous studies have shown that elevated IOP is the primary risk factor for developing the characteristic optic neuropathy associated with this disease (AGIS, 2000; Heijl et al., 2002; Kass et al., 2002; Lichter et al., 2001).

In POAG patients, IOP elevation is due to increased aqueous humor outflow resistance, the majority of which arises from the TM (Tektaş and Lutjen-Drecoll, 2009). Abnormal cell signaling pathway activities damage the TM, increase outflow resistance, and elevate IOP (Fleener et al., 2006; Mao et al., 2010; Shepard et al., 2010; Wang et al., 2008; Wordinger and Clark, 2007; Wordinger et al., 2007, 2008).

One of the cell signaling pathways involved in glaucoma pathogenesis is the Wnt signaling pathway (Wang et al., 2008). In our previous study, elevated *SFRP1* expression was observed in 6 GTM cell strains compared to 6 NTM cell strains at both mRNA and protein levels (Wang et al., 2008). The Wnt pathway is involved in proliferation, development, and homeostasis (Logan and Nusse, 2004). Because of the importance of the Wnt signaling pathway, Wnt signaling activity is under fine control. Otherwise, diseases may result from dysregulated Wnt signaling activity. For example, constitutively activated Wnt signaling is associated with many cancers, while in patients with low bone marrow mass and osteoporosis-pseudoglioma syndrome, this pathway is often suppressed (Levasseur et al., 2005). As well as cancers and bone diseases, the Wnt pathway is a causative factor for a range of ocular diseases including inherited retinal degenerations, Grave's ophthalmopathy, keratoconus, as well as POAG (Mao et al., 2010). We also reported that the secreted Wnt signaling pathway inhibitor *SFRP1* is elevated in GTM cells (Wang et al., 2008). In addition, exogenous SFRP1 induced ocular hypertension in both *ex vivo* perfused human anterior segments and mouse eyes *in vivo*. More importantly, this induction could be blocked by a Wnt pathway activator, which further supports the critical role of SFRP1 and the Wnt pathway in glaucoma. The SFRP1 protein is structurally similar to the Wnt pathway receptor Frizzled but lacks the transmembrane and intracellular domains (Finch et al., 1997; Uren et al., 2000). SFRP1 is able to bind to the pathway activator Wnt, which sequesters Wnt proteins and therefore down-regulates the Wnt signaling activity (Uren et al., 2000).

Although the mechanism(s) responsible for *SFRP1* elevation in GTM cells is unknown, it is clear that DNA methylation-mediated *SFRP1* repression exists in a number of tumor cells (Bovolenta et al., 2008; Rubin et al., 2006). DNA methylation-mediated gene repression functions in both prokaryotes and eukaryotes (Baylin and Ohm, 2006; Jones and Laird, 1999). The 5' position of the cytosine pyrimidine ring can be methylated by DNA methyltransferases (DNMTs). In mammals, DNA methylation usually occurs at the cytosine in CpG dinucleotides. Most of the CpG dinucleotides in the promoter region are in clusters close to the transcriptional start site. Therefore, they are also called CpG islands. Most mammalian promoters are not methylated, which permits gene expression. However, many tumor suppressor genes, including *SFRP1*, are repressed due to the methylation of their CpG islands (Fukui et al., 2005; Suzuki et al., 2002). Lack of *SFRP1* leads to aberrantly high Wnt

signaling activity, unlimited cell proliferation, and tumor formation. On the other hand, *SFRP1* expression can be restored by CpG island demethylation (Suzuki et al., 2002).

Since epigenetic promoter methylation regulates *SFRP1* expression in tumor cells, we determined whether DNA methylation is also a key regulator of *SFRP1* expression in NTM and GTM cells.

2. Material and methods

2.1. Cell culture

HTM cell strains were a kind gift from Alcon Research, Ltd. (Fort Worth, TX). Two NTM cell strains and two GTM cell strains were cultured in DMEM-low glucose medium supplemented with 2 mM glutamine, streptomycin, penicillin (Thermo-scientific, Worcester, MA), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were passaged with Cytodex-3 microcarrier beads (Sigma–Aldrich, St. Louis, MO) as previously described (Steely et al., 1992). Confluent HTM cells in 35 mm dishes or 12-well plates or equivalent were cultured for an additional 7 days before any manipulation. Confluent TM cultures, instead of subconfluent ones were used because a) we tried to mimic *in vivo* conditions, under which the TM cells are confluent; and b) we found that the expression of *SFRP1* in TM cultures is significantly affected by their confluence (Wang et al., 2008), and inconsistent cell confluence may dramatically affect the results. HTM cell donor information is listed in Table 1. The human lung cancer cell line, NCI-H460, was purchased from ATCC (Manassas, VA). NCI-H460 cells were cultured with the same medium as HTM cells.

2.2. AZA-dC treatment

Confluent HTM cells cultured for an additional 7 days in 35 mm or 12-well plates or equivalent were treated with or without the DNA methylation inhibitor, AZA-dC (Sigma) at 2 μ M for 4 days.

Alternatively, HTM cells cultured to 80% confluent in 6-well plates were treated with or without AZA-dC at 2 μ M for 4 days. At the end of treatment, cells in all the wells appeared to be confluent.

2.3. gDNA and RNA isolation

gDNA and RNA were extracted from HTM cells cultured in 35 mm dishes with the Illustra TriplePrep Kit (GE HealthSciences, Piscataway, NJ) according to manufacturer's instructions. Alternatively, the RNAqueous Kit (Ambion, Austin, TX) or RNeasy Micro Kit (Qiagen, Valencia, CA) was used to extract RNA only.

2.4. MS-HRM analysis

100–500 ng gDNA from HTM cells as well as methylated and unmethylated control gDNA (Millipore, Billerica, MA) were bisulfite-converted with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) or Cell-to-CpG Bisulfite conversion kit (Invitrogen). MS-HRM was performed with a reaction mixture (25 μ l) containing 2.5 μ l 10 \times PCR buffer (Sigma), 1.25 μ l 25 mM MgCl₂, 0.4 μ l JumpStart Taq (Sigma), 0.5 μ l 10 mM dNTPs

(Promega, Madison, WI), 1.25 μ l DMSO, 5 μ l 5M betaine (Fisher Scientific, Pittsburgh, PA), 0.1 μ l 0.1 mM forward/reverse primer, 1.25 μ l 20 \times EvaGreen (Biotium, Hayward, CA), and 0.37 μ l of the reference dye Rox. The thermoprofile was 95 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 30 s, consisting of 40 cycles followed by a dissociation curve. *SFRP1* primers were designed by Dr. Jim Herman at Johns Hopkins University (Maryland, MD). The MS-HRM study was performed in the MX-3000p thermocycler (Stratagene, Santa Clara, CA). The primers amplify a gDNA region from -25 to +161 relative to the transcriptional start site.

Primer sequences were:

Forward primer 5' GAGGGTTYGGTYGTAGGAGTTT 3'

Reverse primer 5' CCCCACCAATAACRACCCT 3' (Licchesi et al., 2008)

2.5. qPCR

After DNase I treatment, 2 μ g total RNA was used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems, Carlsbad, California) in 20 μ l reaction mix. qPCR was performed with 1 μ l cDNA. Protocols were the same as for DNA methylation determination. The expression of *SFRP1* was normalized to *GAPDH* with the Ct method. All primers were designed so that they either flank an intron or span an exon-exon junction. The thermoprofile was 95 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 60 s, and 72 $^{\circ}$ C for 30 s, for a total of 40 cycles. PCR primer sequences were:

SFRP1 forward: 5' TCTGAGGCCATCATTGAACA 3'

SFRP1 reverse: 5' TCAGGGGCTTCTTCTTCTTG 3', giving an amplicon of 118 bp.

GAPDH forward: 5' GGTGAAGTCCGAGTCAAC 3'

GAPDH reverse: 5' CCATGGGTGGAATCATATTG 3' (Martens et al., 2003), giving an amplicon of 153 bp.

Because the Ct method compares the relative expression level of one sample to the other, a "reference" sample is required for analysis. To compare *SFRP1* expression levels among the 4 HTM cell strains ($n = 3$ for each cell strain), one of the three GTM198-04 samples was chosen as "reference" and its *SFRP1* level was set at 100%. Then, every other sample (2 + 3 + 3 + 3) was compared to this one to obtain their relative *SFRP1* expression levels for statistical analysis. To compare *SFRP1* expression levels with or without AZA-dC treatment, for each cell strain, an untreated sample was used as "reference" and its *SFRP1* level was set at 100%. Then, every other sample (2 untreated and 3 treated of the same strain) was compared to this one to obtain their relative *SFRP1* expression levels for statistical analysis.

2.6. Data analysis

Data were analyzed using Student's *t*-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Differential expression of *SFRP1* in HTM cells

Our previous studies showed that *SFRP1* expression is elevated in GTM cells (Wang et al., 2008). To validate the TM cell strains used in this study, we compared *SFRP1* expression by qPCR. Four representative cell strains, including two NTM cell strains and two GTM cell strains whose *SFRP1* expression levels were significantly higher than the NTM cells (Fig. 1), were used for further analysis.

3.2. The *SFRP1* promoter of HTM cells was unmethylated

To study whether the differential expression of *SFRP1* in NTM and GTM cells is due to *SFRP1* promoter hypermethylation, we used the MS-HRM method (Lorente et al., 2008; Smith et al., 2009; Wojdacz and Dobrovic, 2007). Briefly, after bisulfite treatment, the CG pairs in unmethylated gDNA are converted to AT pairing, while those in methylated gDNA are preserved. If primers are designed to amplify a region containing CpG islands, PCR products will have different temperature of melting (T_m): methylated gDNA will have higher T_m ; unmethylated gDNA will have lower T_m ; and a mixture of methylated and unmethylated gDNA will show both T_m s. This method is reported to be sensitive enough to detect methylation difference by as few as three CpG sites (Smith et al., 2009).

We chose the CpG islands that have been well-documented and whose methylation status is involved in regulating *SFRP1* expression (Fig. 2A) (Fukui et al., 2005; Jost et al., 2008; Licchesi et al., 2008; Mao et al., 2010; Nojima et al., 2007). We found that PCR products from fully methylated or unmethylated control human *SFRP1* promoter had their T_m at approximately 81 °C and 76 °C, respectively, while all four HTM cell strains showed T_m at 76 °C (Fig. 2B–E, and Table 2). MS-HRM studies also demonstrated high specificity because primer dimers had a small T_m peak at much lower temperature (~73 °C) (Fig. 2F), and gDNA without bisulfite conversion, whether it is unmethylated or methylated, could not be amplified (Fig. 2G).

Besides these four HTM cell strains, we screened additional seven HTM cell strains (5 NTM and 2 GTM) with MS-HRM. Similarly, the seven HTM cell strains all had a T_m peak close to the unmethylated gDNA control (data not shown). Therefore, our results suggest that the CpG islands of the *SFRP1* promoter in HTM cells were all unmethylated.

3.3. Methylation inhibitor treatment did not elevate *SFRP1* expression in HTM cells

Although the CpG islands that we studied were unmethylated, it is possible that other CpG islands within the *SFRP1* promoter are differentially methylated, which could account for differential *SFRP1* expression between NTM and GTM cells. We tested this possibility by treating HTM cells with the methylation inhibitor AZA-dC, to determine whether *SFRP1* promoter demethylation would elevate *SFRP1* expression.

We included a human lung cancer cell line, NCI-H460, as a control since the *SFRP1* promoter of NCI-H460 cells is hypermethylated (Fukui et al., 2005; Licchesi et al., 2008). Furthermore, these hypermethylated CpG islands can be partially demethylated by AZA-dC,

which results in elevated *SFRP1* expression (Fukui et al., 2005; Licchesi et al., 2008). We applied the same AZA-dC regimen to HTM and NCI-460 cells. After AZA-dC treatment, the expression of *SFRP1* was not significantly elevated in any of the four HTM cell strains (each sample in biological triplicates, $p > 0.05$) (Fig. 3). Since DNA methylation may be significantly affected by DNA replication and cell division, it is likely that AZA-dC treatment will have different effects on proliferating HTM cells. We tested this possibility with NTM1054–05 and GTM198–04 cells, the two cell strains with the lowest and highest *SFRP1* expression, respectively (Fig. 1). However, the expression of *SFRP1* was not changed in either cell strain (each sample in biological triplicates, $p > 0.05$) (Fig. 4).

In contrast, the same treatment increased *SFRP1* expression in NCI-H460 cells by more than two fold ($64 \pm 32\%$ vs. $185 \pm 23\%$, $n = 3$, $p < 0.01$) (Fig. 5A). Meanwhile, the *SFRP1* promoters became partially demethylated (Fig. 5B and C).

4. Discussion

In this study, we used the MS-HRM method to detect the methylation status of the *SFRP1* promoter instead of traditional methods like methylation sensitive PCR and bisulfite PCR followed by restriction analysis. If primers are carefully designed, this method is able to simultaneously differentiate methylated DNA, unmethylated DNA, and partially methylated DNA.

By the MS-HRM method, we found the CpG islands in the *SFRP1* promoter were all unmethylated, regardless of TM cell strains or *SFRP1* expression levels. Also, the DNA methylation inhibitor AZA-dC did not significantly alter *SFRP1* expression. These findings suggest that promoter methylation does not play a significant role in regulating *SFRP1* expression in the TM. As a positive control, we also evaluated NCI-H460 cancer cells, where *SFRP1* expression was controlled by promoter methylation.

Combining the data from our previous study (Wang et al., 2008), we found totally 9 out of the 11 GTM cell strains had higher *SFRP1* expression compared to the NTM cell strains with only 6 out of 17 having higher *SFRP1* expression (data not shown), which suggests that the majority of the GTM cells have elevated *SFRP1* expression. The NTM or GTM cell strains in both studies were selected based on the disease history of organ donors. All GTM cell strains were isolated from donors with clinically diagnosed POAG. Although the IOP data of the organ donors are not available, we believe that those POAG patients had elevated IOP because otherwise they would have been diagnosed as normal tension glaucoma. Since there was *SFRP1* dose-dependent IOP elevation in mouse eyes (Wang et al., 2008), it would be very interesting to further investigate whether a similar effect also exists in POAG patients. However, for NTM cells, it is possible that the donors had not developed clinically manifest POAG before death, since age is another important risk factor of POAG. Also, this may explain why some of the NTM cell strains have higher *SFRP1* expression levels.

Epigenetics is defined as a change in gene expression without changing the DNA sequence. One of the most frequently studied epigenetic gene regulation mechanism is DNA methylation. DNA methylation has been extensively studied in development and cancers. In

contrast, the role of this important mechanism has not been investigated in the TM in glaucoma. Although an association between DNA methylation and *SFRP1* expression was not found in this study, we cannot rule out the possibility that DNA methylation plays a role in the regulation of other glaucoma-associated molecules. For example, the expression of TGF- β 2 is elevated in the aqueous humor (Inatani et al., 2001; Picht et al., 2001; Tripathi et al., 1994) and TM (Tovar-Vidales et al., 2011) of glaucoma patients. The expression of the BMP inhibitor, GREMLIN, is also elevated in the glaucomatous TM (Wordinger et al., 2007) and optic nerve head cells (Zode et al., 2009). The mechanism(s) responsible for elevated levels of these molecules is still unclear. In cancer cells, the expression of the key pathway components, TGF- β receptors and GREMLIN, are both affected by DNA methylation (Kang et al., 2010, 1999; Munoz-Antonia et al., 2009; Pinto et al., 2003). Therefore, further studies are required to find out whether other glaucoma-associated molecules are regulated by DNA methylation or other epigenetic mechanisms such as histone acetylation or microRNAs.

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Abbreviations:

AZA-dC	5-aza-2'-deoxycytidine
gDNA	genomic DNA
GTM	glaucomatous trabecular meshwork
HTM	human trabecular meshwork
IOP	intraocular pressure
MS-HRM	methylation sensitive high resolution melting analysis
NTM	normal trabecular meshwork
POAG	primary open angle glaucoma
qPCR	quantitative PCR
SFRP1	secreted frizzled-related protein 1
Tm	temperature of melting
TM	trabecular meshwork

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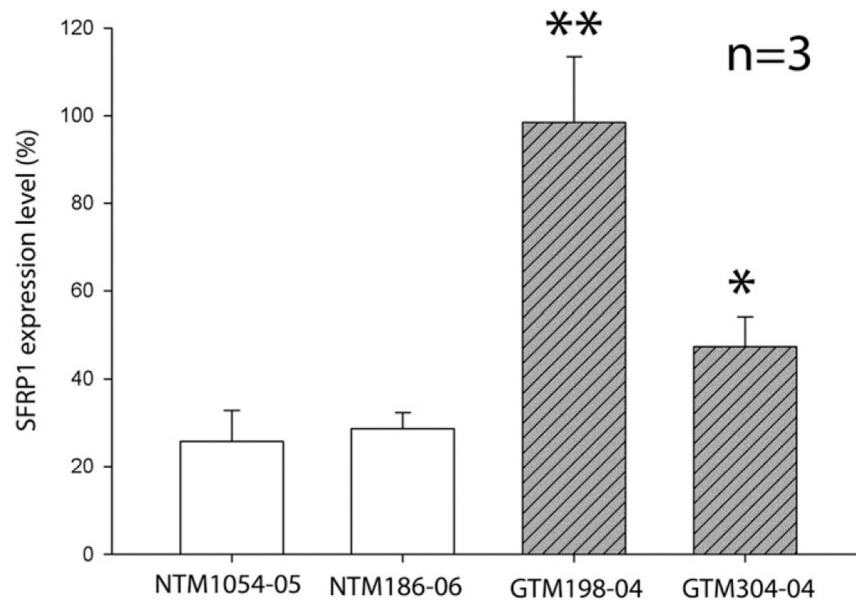


Fig. 1.

Differential expression of *SFRP1* in HTM cells. Four different HTM cell strains were cultured in 35 mm dishes for an additional 7 days after they were confluent. The expression level of *SFRP1* of each cell strain was studied by qPCR. The expression level of *SFRP1* was first normalized to *GAPDH*, and then to a GTM198–04 sample, whose *SFRP1* expression level was set at 100%. All experiments were performed in biological triplicates ($n = 3$). Bars = means \pm standard deviation (SD). *: $p < 0.05$ in comparison with either NTM cell strain. **: $p < 0.01$ in comparison with either NTM cell strain.

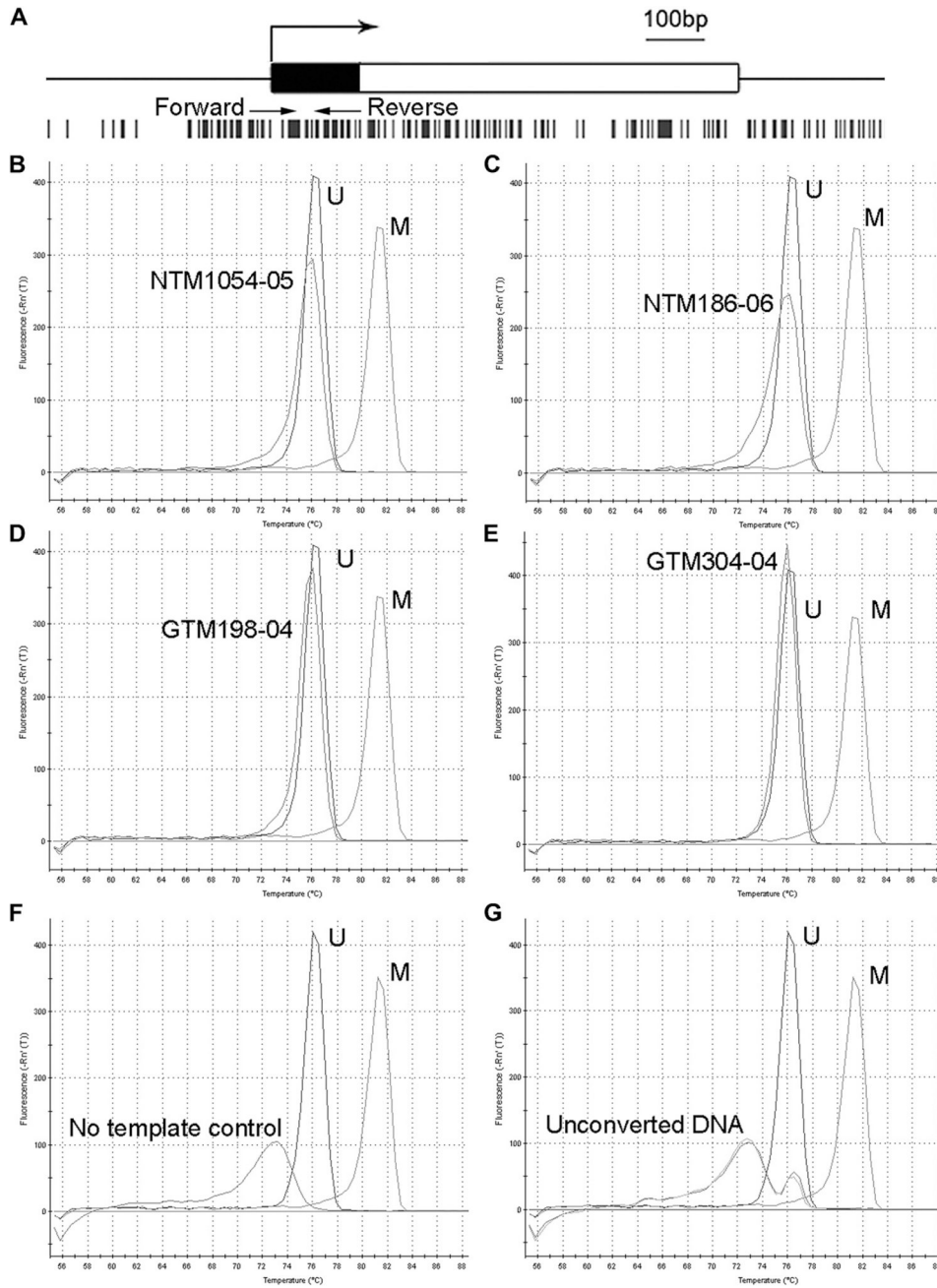


Fig. 2. The methylation status of the *SFRP1* promoter of HTM cells. The CpG islands in the *SFRP1* promoter region are shown in (A). Black bar: the 5' untranslated region. White bar: the coding region. Large arrow: transcriptional start site. Vertical black bars: CpG sites. Small arrows: the primers for MS-HRM. (B–E): MS-HRM analysis of the *SFRP1* promoter of HTM cells. gDNA from the same cells as described in Fig. 1 was used for MS-HRM after bisulfite conversion. The first order derivative of fluorescence intensity was plotted against temperature. Each peak represents the T_m of the PCR product. The difference in the T_m between completely unmethylated control DNA (U) and methylated control DNA (M) was

approximately 5 °C. The T_m of all four HTM cell strains was almost the same as that of the unmethylated control DNA. Negative control studies included PCR reactions without DNA template (No template control) (F), or with unmethylated or methylated DNA that were not subjected to bisulfite conversion (Unconverted DNA) (G). All experiments were performed in biological triplicates, and representative data are shown.

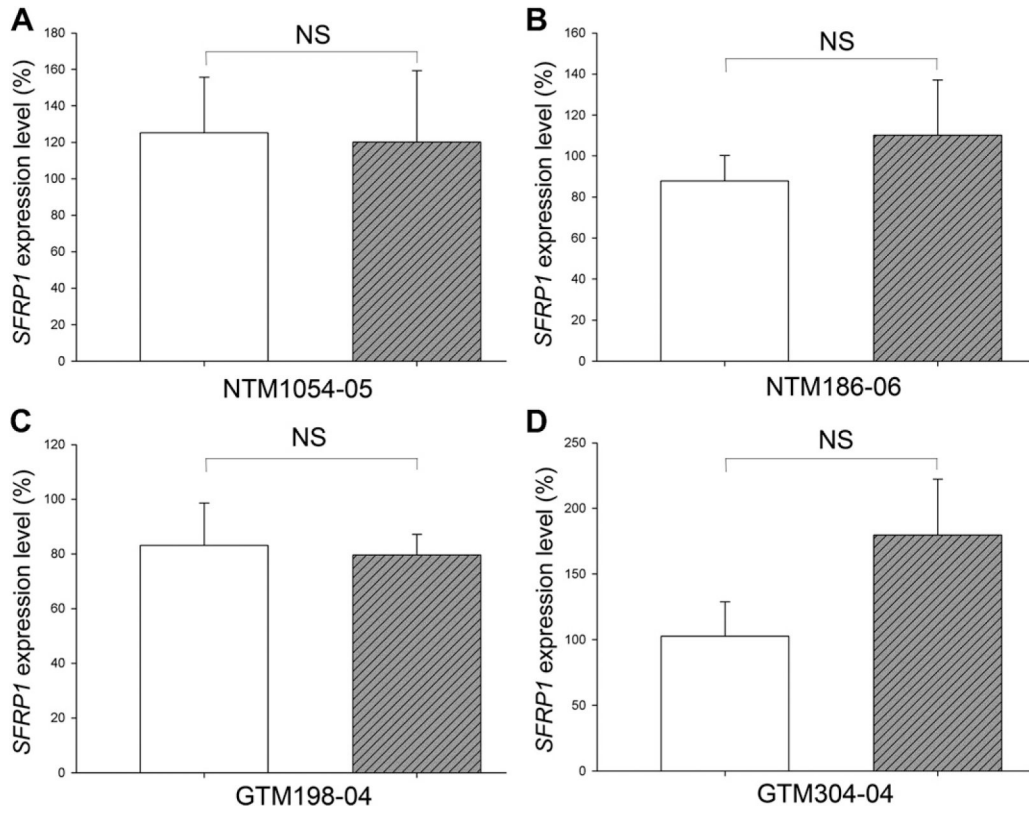


Fig. 3. AZA-dC treatment did not affect *SFRP1* expression in confluent HTM cells. Confluent HTM cells cultured for additional 7 days were subjected to treatment with (hatched bars) or without (open bars) 2 μ M AZA-dC for 4 days. The expression levels of *SFRP1* were studied by qPCR. The expression level of *SFRP1* was first normalized to *GAPDH*, and then to a control sample, whose *SFRP1* expression level was set at 100%. All experiments were performed in biological triplicates ($n = 3$). Bars = means \pm standard deviation (SD). NS: not significant ($p > 0.05$).

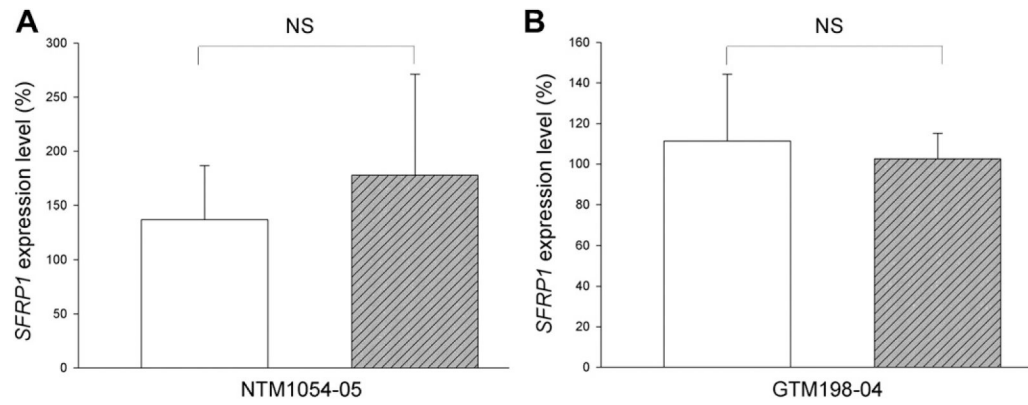


Fig. 4. AZA-dC treatment did not affect *SFRP1* expression in non-confluent HTM cells. Eighty percent confluent HTM cells were subjected to treatment with (hatched bars) or without (open bars) 2 μ M AZA-dC for 4 days. The expression levels of *SFRP1* were studied by qPCR. The expression level of *SFRP1* was first normalized to *GAPDH*, and then to a control sample, whose *SFRP1* expression level was set at 100%. All experiments were performed in biological triplicates ($n = 3$). Bars = means \pm SD. NS: not significant ($p > 0.05$).

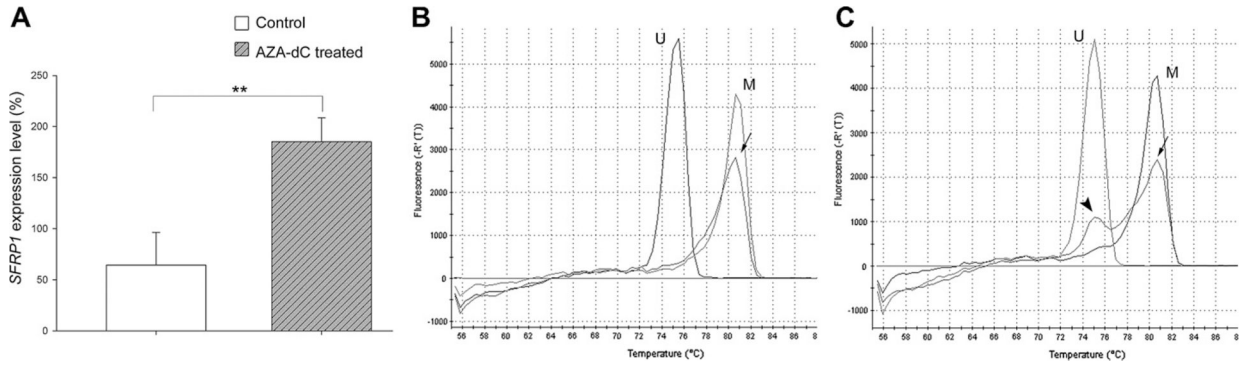


Fig. 5.

AZA-dC treatment induced *SFRP1* expression and demethylation of the *SFRP1* promoter in NCI-H460 cells. NCI-H460 cells were treated with or without 2 μ M AZA-dC for 4 days. (A) The expression level of *SFRP1* of each cell strain was studied by qPCR. The expression level of *SFRP1* was first normalized to *GAPDH*, and then to a control sample, whose *SFRP1* expression level was set at 100%. The experiment was performed in biological triplicates ($n = 3$). Bars = means \pm SD. **: $p < 0.01$. (B–C) MS-HRM studies showed that NCI-H460 cells without AZA-dC treatment had methylated *SFRP1* promoters (B, arrow). AZA-dC treatment caused demethylation of the *SFRP1* promoter in some cells, which resulted in two peaks on the melting curve (C). The major peak (arrow) represented the methylated promoters while the minor peak (arrowhead) represented the unmethylated promoters. U: unmethylated DNA control. M: methylated DNA control.

Table 1

HTM cell donor information.

Cell strain	Age	Sex	Passage
NTM1054-05	58	F	6
NTM186-06	80	F	6
GTM198-04	80	M	9
GTM304-04	75	F	7

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Table 2The T_m of the HTM cell strains.

Cell strains	T _m (°C)		N
	Mean	SD	
NTM1054-05	76.02	0.24	3 ^a
NTM186-06	76.02	0.24	3 ^a
GTM198-04	76.18	0.24	3 ^a
GTM304-04	76.35	0.00	3 ^a
Methylated DNA control	81.50	0.25	3 ^b
Unmethylated DNA control	76.18	0.24	3 ^b

^aBiological replicates.^bTechnical replicates.

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