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Genetic and epigenetic mechanisms combine to control MMP1 expression and its association with preterm premature rupture of membranes

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Degradation of fibrillar collagens is believed to be involved in the rupture of the fetal membranes during normal parturition and when the membranes rupture prematurely. Matrix metalloproteinase 1 (MMP1) is a key enzyme involved in extracellular matrix turnover, and genetic variation in the *MMP1* promoter is associated with the risk of preterm premature rupture of membranes (PPROM). We determined whether epigenetic factors contribute to the control of MMP1 expression in the human amnion. Inhibition of DNA methylation with 5-aza-2′-deoxycytidine in amnion fibroblasts resulted in significantly increased *MMP1* gene transcription, and an associated significant increase in MMP1 production. These effects were correlated with reduced DNA methylation at a particular site (−1538) in the *MMP1* promoter. DNA methylation at this site in amnion was reduced in a larger percentage of fetal membranes that ruptured prematurely. A new T > C single nucleotide polymorphism (SNP) [AF007878.1 (MMP1):g.3447T>C] in the *MMP1* promoter was also identified. The minor C allele was always methylated *in vivo*, and when methylated, resulted in increased affinity for a nuclear protein in amnion fibroblasts. The minor C allele had reduced promoter activity as assessed by plasmid transfection studies and chromatin immunoprecipitation assays using amnion fibroblasts heterozygous for the T > C SNP. In a case–control study, the minor C allele was found to be protective against PPROM, consistent with its reduced promoter function. We conclude that in addition to genetic variation, DNA methylation plays a role in controlling MMP1 expression and risk of an adverse obstetrical outcome.

INTRODUCTION

Extracellular matrix homeostasis is a key process in maintenance of the tensile strength of the fetal membranes, the tissue that encapsulates the fetus and amniotic fluid, isolating this compartment from the external world (1). The integrity of the fetal membranes is secured mostly by fibrillar collagens. Preterm premature rupture of the membranes (PPROM), which occurs when the strength of the membranes is compromised, is defined as rupture before 37 completed weeks of gestation. PPROM occurs in 30–40% of preterm deliveries, and thus is the leading identifiable cause of preterm birth and its complications (1). Although the underlying mechanisms of PPROM are still poorly understood (2),

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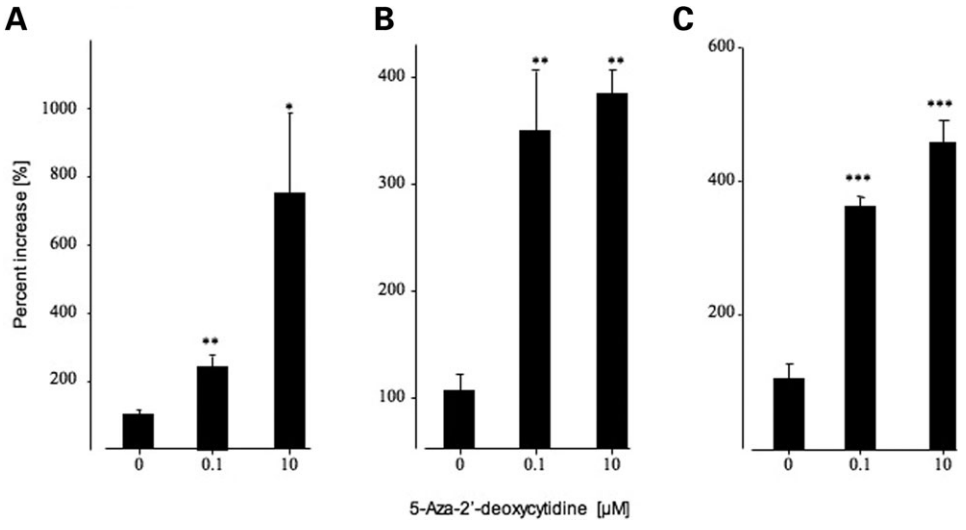

Figure 1. 5-Aza treatment causes increased *MMP1* gene transcription and matrix metalloproteinase 1 (MMP1) production. Cultures of amnion fibroblasts were treated with control medium containing 0.1 or 10 μM 5-Aza for 72 h. Nascent MMP1 transcripts (**A**) and MMP1 mRNA (**B**) were quantitated by quantitative real-time polymerase chain reaction. MMP1 in the medium (**C**) was measured by enzyme-linked immunosorbent assay. Values are means ± SE from three separate experiments with triplicate cultures in each treatment group normalized to the no-treatment groups. Asterisk (*) indicates values that are significantly different from each other by the Tukey-Kramer test, $P < 0.05$.

reduced fetal membrane collagen synthesis or increased collagen degradation are thought, but not proven, to contribute towards membrane rupture (3). Consequently, there is considerable interest in understanding how the expression and activity of matrix degrading enzymes are regulated in the fetal membranes both in normal parturition and when the membranes rupture prematurely.

Matrix metalloproteinase 1 (MMP1), the major collagenase elaborated by fibroblasts, has been implicated in a variety of developmental, physiological and pathological processes including rupture of the fetal membranes during parturition (4, 5), tumor invasion (6, 7) and tissue remodeling/destruction associated with inflammation (8, 9). MMP activity is determined by levels of gene expression, proenzyme activation and the action of endogenous inhibitors of metalloproteinases (10).

Variation in gene expression has been linked to a polymorphism in the *MMP1* promoter and this variation has been proposed to have functional significance. A single nucleotide polymorphism (SNP), referred to in the literature as −1607 1G or 2G allele (rs1799750:>G; AF007878.1(MMP1):g.2775delG) was found to significantly affect *MMP1* promoter function with the 2G variant having greater promoter activity (4). The promoter 2G allele was associated with increased risk of metastasis of certain solid tumors (4) and increased risk of preterm premature rupture of membranes (4), processes associated with degradation of the extracellular matrix.

In addition to genetic variation, epigenetic factors could influence levels of MMP expression. However, little is known regarding the potential epigenetic control of MMP1 expression. Here, we describe variation in cytosine methylation in the *MMP1* promoter, which is associated with altered promoter function and gene expression. We discovered that hypomethylation of a cytosine at −1538 is associated with increased MMP1 expression and risk of PPROM, and that the C allele of a AF007878.1(MMP1):g.3447T>C SNP, which is always methylated, reduces promoter activity and *MMP1* gene transcription, and protects against PPROM. Thus, both genetic (genomic DNA sequence variation) and epigenetic (DNA methylation) factors combine to determine MMP1 expression and influence the association with PPROM.

RESULTS

Variable patterns of matrix metalloproteinase 1 promoter methylation

To determine if DNA methylation affects MMP1 expression, we treated cultures of amnion fibroblasts with 0.1 and 10 μM 5-aza-2′-deoxycytidine (5-Aza), a DNA methyl transferase inhibitor, to block DNA methylation for 72 h, and measured MMP1 released into the culture media by enzyme-linked immunosorbent assay (ELISA). The DNA methyl transferase inhibitor increased MMP1 production (Fig. 1). In addition, MMP1 nascent transcripts and MMP1 mRNA were increased in a similar pattern (Fig. 1), indicating that increased *MMP1* gene transcription caused the increase in MMP1 release. We performed bisulfite sequence analysis on control cells and cells treated with 5-Aza using primers that permitted analysis of the 14 potential methylation sites shown in Table 1, and discovered that there was a consistent reduction in DNA methylation at site −1538 (Fig. 2), which could also be detected using a simple combined bisulfite restriction analysis (COBRA) in which DNA was treated with bisulfite and then subjected to restriction enzyme digestion with HpyCH4. A reduction in DNA methylation at this site was seen after 24 h of culture in the presence of 5-Aza and maintained for subsequent days of culture, which paralleled production of MMP1 protein (Fig. 3). The consistent finding of reduced methylation at the −1538 site associated with increased MMP1 expression suggested that this is an important region for epigenetic control of *MMP1* transcription.

Table 1. Polymerase chain reaction primer sequences

	Forward primer	Reverse primer
1	5′-tgatgtttttgagaagaggatttttt-3′	5′-tttcaacactttcctcccctta-3′
	Nested 5′-tgttatgttatttagatgaggaaattg-3′	5′-tccccttataaattcctattttcttt-3′
2	5′-ggaaggagagtggggtatgag-3′	5′-atttatacattcctccattcaaaaa- 3′
	Nested 5′-tggggtatgagtaggggagtt-3′	5′-catctcctacccattaaaataaataca-3′
3	5′-ttatttttaatgggtaggagatgttaaa-3′	5′-aaaacaaataaaatccttccctaaa-3′
	Nested 5′-tttttgaatggaggaatgta-3′	5′-tccttccctaaaacttatctaaa-3′
4	5′-aaggggtggggagttattt-3′	5′-ttaaccaaacataaaatcatacaccta-3′
	Nested 5′-gggtggggagttattttatattt-3′	5′-catacacctataatcctaatcactaaaaa-3′
5	5′-tttaggttgattttgaatttttgg- 3′	5′-cacaccttactcccaaaacaaa-3′
	Nested 5′-tttttgggtttaagtgattttt-3′	5′-caactcccccaacactc-3′
6	5′-agtttaataaaggtagaagggaatttt-3′	5′-caataattttctccaattcatttcttt-3′
	Nested 5′-aaaggtagaagggaattttagagaa-3′	5′-ctccaattcatttctttcctattta-3′
7	5′-ggaaagaaatgaattggagaaaa- 3′	5′-ttcccaacctcttactactcca-3′
	Nested 5′-ggaaagaaatgaattggagaaaa-3′	5′-aaaatccttacccttccaaaaa-3′

To determine if there is natural variation in the methylation status of the *MMP1* gene proximal promoter, and in particular, variation at the −1538 site identified in the *in vitro* experiments using 5-Aza, which could contribute to altered gene expression, we conducted a preliminary analysis of DNA methylation in genomic DNA extracted from 10 randomly collected amnion specimens. We found that the *MMP1* promoter in amnion was fairly highly methylated, an observation that was somewhat surprising given that the gene is expressed in amnion and, therefore, would be expected to have a relatively un-methylated promoter (Fig. 4). However, the extent of methylation at specific sites varied among samples. Interestingly, two amnion samples had substantially reduced methylation at the −1538 site when compared with the other eight specimens. The limited number of samples evaluated in this study did not permit any statistically significant conclusions regarding the variation in DNA methylation at specific sites. However, the results demonstrate that there are discernable differences in *MMP1* promoter DNA methylation among amnion samples, which include variation in methylation at the −1538 site.

To follow-up on the observations that 5-Aza treatment causes increased MMP1 expression in association with a change in DNA methylation at −1538 and that in amnion samples this site is variably methylated, we used the COBRA analysis to examine methylation at −1538 in a larger group of amnion samples from normal term deliveries and amnions from pregnancies complicated by PPROM (Fig. 5). Examining the distribution of samples according to the determined percentage of methylated DNA at −1538, we found that the mean percent of DNA methylation for normal term pregnancies and PPROM pregnancies were around 78%, but that there was considerable prevalence of lower DNA methylation in the PPROM group. A statistical test for differences in distribution was conducted using the Wilcoxon Rank sum test, which indicated a statistically significantly difference between the PPROM and control amnion samples ($P < 0.037$).

Identification of a functional AF007878.1(MMP1):g.3447T>C single nucleotide polymorphism in the matrix metalloproteinase 1 promoter in which the C allele is methylated

Sequence analysis of the *MMP1* promoter fragment amplified by polymerase chain reaction (PCR) from more than 30 unrelated African-Americans identified a T>C SNP, with 'T' being the major allele.

We performed promoter function studies to compare the activities of the two alleles in the primary cultures of human amnion fibroblasts (Table 2). Previous studies identified a AF007878.1(MMP1):g.2775delG 2G allele which creates a new Ets transcription binding site that increases *MMP1* promoter activity. We therefore examined the impact of the AF007878.1(MMP1):g.3447T>C SNP on a background of the *MMP1* 2G promoter allele. The activity of the *MMP1* promoter with the minor C allele is 30% lower when compared with the activity of the T allele ($P < 0.05$).

Methylation status of the AF007878.1(MMP1): G.3447T>C C allele

The MMP1 promoter was analyzed for DNA methylation by bisulfite sequencing and COBRA analysis. Analysis of more than 15 samples of genomic DNA revealed that whenever the C allele was present, it was methylated, raising the possibility that epigenetic modification influences the functional significance of the C allele. Since the promoter analyses were conducted with constructs that were not methylated, which was verified by bisulfite sequence analysis, these differences in promoter activity do not reflect the impact of methylation. Therefore, we conducted additional studies to evaluate the function of the endogenous promoter alleles and the affinity of non-methylated and methylated oligonucleotides representing the *MMP1* promoter sequence.

Methylation of the AF007878.1(MMP1):G.3447T>C C allele augments the binding of amnion fibroblast nuclear proteins

In vitro assays of protein–DNA interactions were used to determine if specific protein complexes bind to the AF007878.1(MMP1):g.3447T>C SNP in the *MMP1* promoter. We analyzed the binding of nuclear extract (NE) proteins prepared from the primary cultures of human amnion fibroblasts (Fbs) to oligonucleotides representing the T allele, unmethylated C allele and methylated C allele by electrophoretic mobility shift assay (EMSA) (Fig. 6). Specific binding of proteins was identified through cross-competition using unlabeled oligonucleotides representing the T and C alleles and an irrelevant nucleotide sequence. The EMSA results revealed two specific complexes with NE proteins regardless of the genotypes. Probes containing the minor C allele had increased affinity for these nuclear proteins compared with the probe containing the major T allele. Furthermore, an oligonucleotide probe containing the methylated C allele bound substantially more of the NE proteins compared with the unmethylated C oligonucleotide probe.

Based on the fact that the C allele reduced *MMP1* promoter activity, these gel shift results suggested that a repressor of transcription in fibroblast cells binds with higher affinity to the C allele, which could account for decreased *MMP1* promoter activity of the C allele. The findings also suggested that the endogenous methylated C allele would have an even greater effect on promoter activity, which could be revealed through

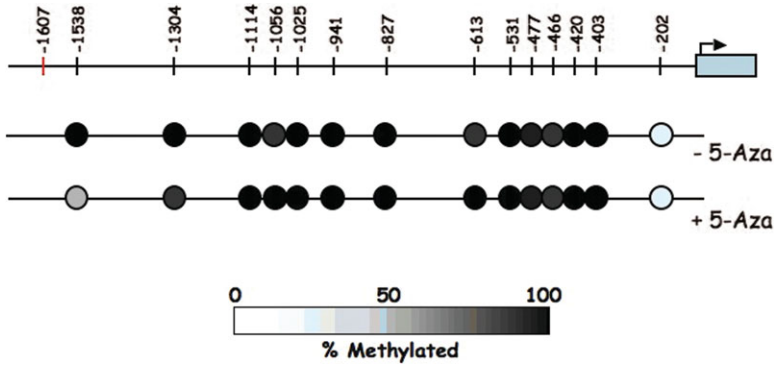

Figure 2. 5-Aza-induced changes in DNA methylation of the *MMP1* promoter. Amnion fibroblasts were treated with control medium or medium containing 10 μM 5-Aza for 72 h. Genomic DNA was isolated and then subjected to bisulfite sequencing. The percent DNA methylation was determined by the analysis of 8–10 PCR products from at least two different bisulfite treatments of genomic DNA samples.

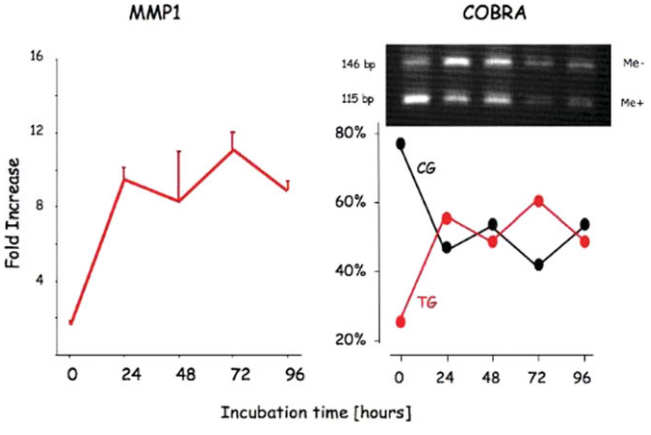

Figure 3. Time-dependent changes in matrix metalloproteinase 1 (MMP1) production and DNA methylation at site −1538 of the *MMP1* promoter. Amnion fibroblasts were treated with control medium or medium for 96 h containing 10 μM 5-Aza for the indicated times. Media were changed every 24 h and samples were analyzed from the last 24 h of culture. DNA methylation was determined by combined bisulfite restriction analysis (COBRA) and MMP1 released into the culture medium was quantitated by enzyme-linked immunosorbent assay. The ethidium bromide-stained gel shows digested polymerase chain reaction products representing the methylated (115 bp, Me+) and unmethylated (146 bp, Me−) DNA from individual time-points, with the percentage of methylated (CG) and unmethylated (TG) DNA displayed below the gel in graphical form. The results shown are from a representative experiment in which cells were pooled from three- to six-wells for each point. MMP1 was measured in individual wells and results presented as the fold increase over control cultures with means ± SD.

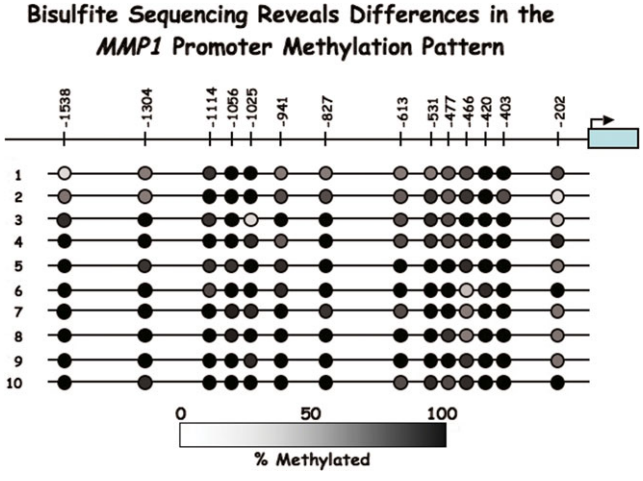

Figure 4. DNA methylation of the *MMP1* promoter in amnions. Ten different amnions were collected and subjected to bisulfite DNA sequence analysis as described in the text. Percent methylation was determined using a series of polymerase chain reaction products.

the analysis of *MMP1* gene expression and transcriptional activity *in vivo*.

Influence of AF007878.1(MMP1):G.3447T>C C single nucleotide polymorphism on matrix metalloproteinase 1 nascent transcript and mRNA levels

To determine if the T>C SNP influences endogenous *MMP1* promoter function, which is reflected by increased levels of MMP1 nascent transcripts and mRNA, quantitative reverse transcriptase (RT)-PCR was carried out on nucleic acids extracted from primary cultures of human amnion fibroblasts. All of the cultured cells were homozygous for the −1607 2G allele. Quantitative PCR revealed that MMP1 nascent transcript levels are more than 4-fold reduced in samples heterozygous for the C>T SNP compared with samples homozygous for the major T allele ($P = 0.006$) (Fig. 7). Similarly, mRNA levels were more than 3-fold lower in samples heterozygous for the C>T SNP when compared with those with a T/T genotype ($P = 0.003$). While these results are in agreement with the promoter studies, they demonstrate a more profound effect of the C allele. This discrepancy in the magnitude of the effect could be explained by methylation of the endogenous C allele.

The AF007878.1(MMP1):G.3447T>C C allele is associated with reduced matrix metalloproteinase 1 gene transcription as determined by chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was used to identify the regulatory function of the T>C SNP in the *MMP1* promoter. ChIP assays detect the functional association of DNA variation with expression by examining the binding of the transcriptional machinery of a gene and its correlation with

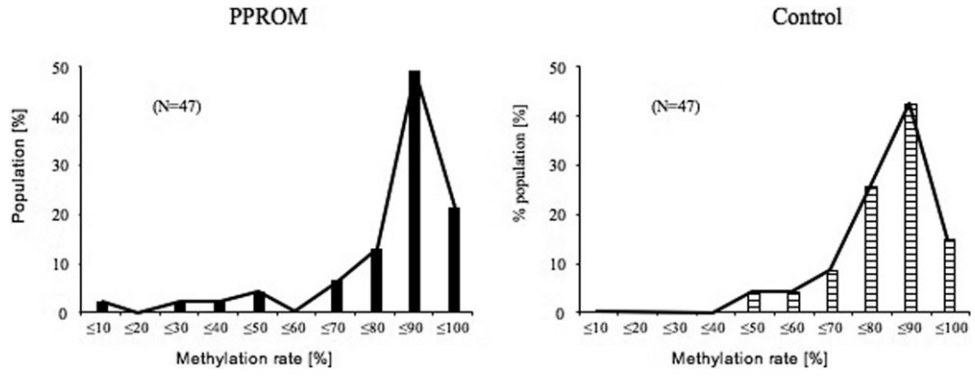

Figure 5. Distribution of the methylation pattern at position −1538 in the *MMP1* promoter amnion genomic DNA prepared from normal term delivery and preterm premature rupture of membranes specimens. Genomic DNA was isolated and subjected to combined bisulfite restriction analysis to determine methylation status as described in the text.

Table 2. AF007878.1(MMP1):g.3447T>C genotype-dependent promoter activity

Construct	
pGL3-basic	0.64 ± 0.02^{a}
MMP1-2G/T	270.5 ± 31.0^{b}
MMP1-2G/C	204.6 ± 28.96^{c}

Cultured amnion fibroblasts were transfected with the indicated plasmids and a *Renilla* plasmid to control for transfection efficiency as described in the text. The value represents the means ± SE from three separate experiments with triplicate wells for each treatment. Values with different superscripts are significantly different ($P < 0.05$) from each other by the Tukey-Kramer test.

DNA variation at the locus. The allelic differences of T>C in transcriptional activity of MMP1 were measured by comparing the amounts of Pol II bound to chromatin for each allele in amnion fibroblasts heterozygous for the T>C SNP. Again, all samples were homozygous for the AF007878.1(MMP1):g.2775delG 2G allele. An antibody to RNA polymerase II was used to isolate chromatin cross-linked with Pol II (Fig. 8). If the T and C alleles were transcribed at an equal rate, we would expect to find a 1:1 ratio for each allele in immunoprecipitated DNA. Deviation from this ratio reflected greater or lesser transcription. The result of T>C input controls ($n = 3$) showed that the allelic ratios were, as expected, close to 1 in genomic DNA (ratio of T>C: 0.93 ± 0.07). However, the major T allele was precipitated to a greater extent with the anti-RNA polymerase II antibody ($P = 0.001$, ratio T/C: 2.24 ± 0.13), which indicates more active transcription of the T allele.

A case–control study reveals that the AF007878.1(MMP1): G.3447T>C C allele protects against preterm premature rupture of membrane

To test the possible association between the T>C SNP and PPROM, we performed a case–control study of neonatal genotypes from normal term pregnancies ($n = 361$) and pregnancies complicated by PPROM ($n = 284$) in an African-American population, which was selected because PPROM is two times more prevalent in African-Americans (1, 11, 12). The study focused on the genotype of the offspring based on the hypothesis that genotype of the extraembryonic tissues (fetal membranes) represents the primary determinant of risk of premature rupture of the membranes. Based on an analysis of 29 ancestry informative markers, the cases and controls had, using a dihybrid model of admixture, equivalent African ancestry ($P > 0.05$).

There were no significant differences in maternal age, gravidity and parity, but the length of gestation and birth weight were significantly lower in the PPROM group, as expected, compared with controls. The demographic characteristics of controls, neonates born at term from normal pregnancies and cases, neonates from pregnancies complicated by PPROM, are shown in Table 3.

The minor C allele was found to be significantly protective against PPROM compared with the major T allele (OR, 0.7451; CI, 0.563–0.984; $P = 0.0326$). The number of copies of SNP T>C alleles was significantly associated with PPROM ($P = 0.0117$). Neonates who were homozygous for the major T allele had 3.51-fold higher risk for PPROM when compared with neonates who were homozygous for the minor C allele ($P = 0.007$). The OR for PPROM for neonates who were heterozygous for the T>C SNP was reduced by 15.9%, but did not achieve statistical significance.

DISCUSSION

MMP1 is the major collagenase produced by fibroblasts that initiates the process of degradation of fibrillar collagens (10). Given its key role in the turnover of the extracellular matrix, the factors that control MMP1 expression and activity are a subject of considerable interest both with respect to normal physiology as well as in pathological states where collagen degradation is inappropriate. Evidence has been previously reported that genetic variation in the *MMP1* promoter influences gene expression and is associated with pathophysiological processes such as tumor metastasis and PPROM (4). Here, we describe another level of regulation, namely, control of expression through DNA methylation and the interaction between genetic variation in the *MMP1* promoter sequence and DNA methylation. Collectively, these findings

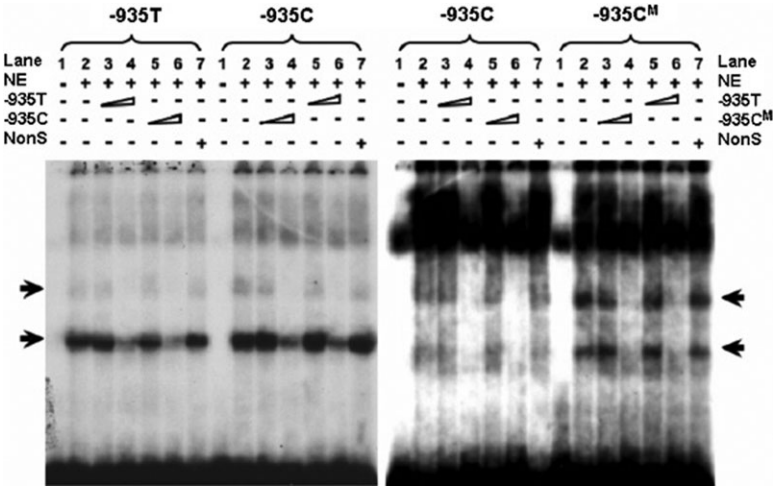

Figure 6. The MMP1 promoter region containing the AF007878.1(MMP1):g.3447T>C single nucleotide polymorphism (−935 C>T SNP) specifically binds a protein in the nuclear extract from primary cultures of human amnionic fibroblasts. Electrophoretic mobility shift assay was performed comparing the binding affinity of T allele probe and unmethylated C allele probes (left) or the binding ability of unmethylated C allele probe and methylated C allele probe (right). Specific binding was established by the addition of increasing amounts of unlabeled double-strand oligonucleotides representing the three MMP1 alleles or an irrelevant nucleotide sequence. Arrowheads indicate specific protein–DNA complexes.

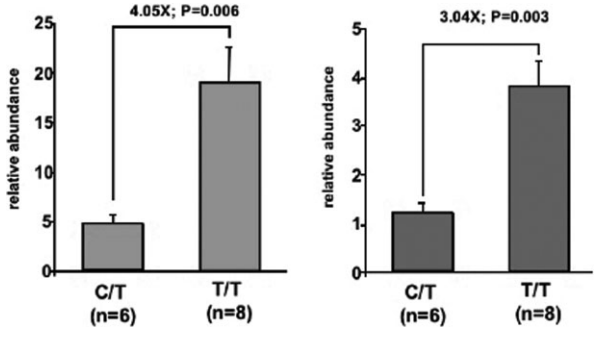

Figure 7. *In vivo* expression of *MMP1* in amnion depends on the AF007878.1(MMP1):g.3447T>C genotype. Quantitative real-time polymerase chain reaction analysis of *MMP1* nascent transcripts (left panel) and mRNA (right panel) in amnions homozygous for the T allele or heterozygous for the T>C SNP. All amnions were homozygous for the AF007878.1(MMP1):g.2775delG 2G allele. Values are means ± SE from the indicated number of samples analyzed.

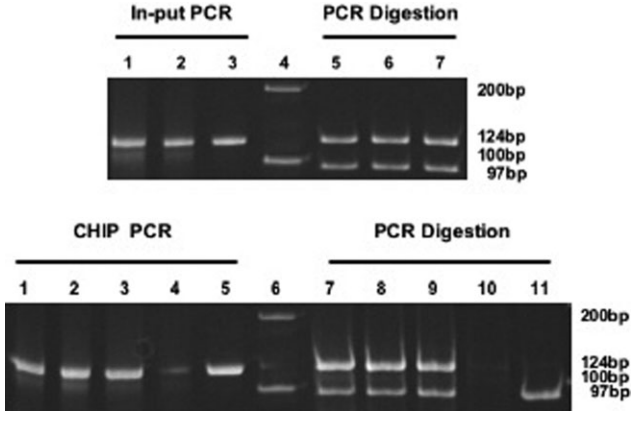

Figure 8. Chromatin immunoprecipitation (ChIP) analysis of the impact of the AF007878.1(MMP1):g.3447T>C SNP in cultures of amnion fibroblasts. The upper panel shows the input DNA polymerase chain reaction (PCR) amplification of the MMP1 promoter fragment containing T>C SNP and the digested PCR product. Lanes 1, 2 and 3 show the 124 bp PCR product from the input from three different immunoprecipitations, each from a different culture. Lane 4 is 100 bp DNA marker. Lanes 5–7 show the PCR digestion products of the three different samples shown in lanes 1–3. The lower panel shows the PCR amplification and digestion results of ChIP DNA. Lanes 1–3 show the PCR products of the samples described above released from the anti-RNA polymerase II immunoprecipitation. Lane 4 is the PCR product of DNA linked with IgG immunoprecipitation. Lane 5 is the PCR of MMP1 plasmid DNA containing the C allele. Lane 6 is 100 bp DNA marker. Lanes 7–9 show the PCR digestion products of the samples shown in Lanes 1–3. Lane 10 is the PCR digestion result of Lane 4. Lane 11 is the PCR digestion of C allele plasmid DNA.

demonstrate the combinatorial influence of genetic and epigenetic factors in determining gene expression levels and their impact on physiology and pathophysiology.

Our studies on cultured amnion fibroblasts, the cells that lay down the fibrillar collagen of the amnion, revealed that inhibition of DNA methylation results in increased *MMP1* gene expression, which was associated with selective alterations in the methylation of cytosines, and in particular, reduced methylation at a single site in the promoter. This observation suggests that region-specific alterations in DNA methylation have an important influence over *MMP1* gene transcription. However, the mechanism by which methylation at the −1538 site alters gene transcription remains to be elucidated. DNA methylation can interfere with the binding of transcription factors, and the association of reduced DNA methylation at the −1538 site with increased MMP1 expression might, therefore, suggest that relative hypomethylation of the −1538 site results in increased binding of a transcriptional activator. However, as demonstrated in our studies on the AF007878.1(MMP1):g.3447T>C C allele, DNA methylation can increase specific binding of nuclear factors, including members of the methyl DNA binding proteins and this may lead to diminished gene transcription.

Table 3. Demographic characteristics and clinical outcomes of index pregnancies.

	Controls ($n = 361$)	PPROM ($n = 284$)	*P*-value
Maternal age (years)	26 ± 4.6	25 ± 5.8	0.63
Gravidity	3.1 ± 1.9	3.4 ± 2.1	0.34
Parity	1.4 ± 1.5	1.5 ± 1.6	0.55
Gestational age (weeks)	39.1 ± 1.3	31.9 ± 2.8	<0.0001
Birth weight (g)	3309 ± 483	1937 ± 502	<0.0001

Values presented are the means $\pm$ SD. PPROM, preterm premature rupture of membranes.

The -1538 site was variably methylated in amnion samples we analyzed and a significantly greater number of amnion samples collected from women whose pregnancies were complicated by PPROM had reduced methylation at this site when compared with amnions collected from women who had normal term pregnancies. Since hypomethylation at -1533 was associated with increased MMP1 expression by 5-Aza-treated amnion fibroblasts, we suggest that the increased number of amnion samples with hypomethylation at -1538 from PPROM reflects increased MMP1 expression *in vivo* that promotes unscheduled fetal membrane rupture.

During the course of these studies, we also discovered a new promoter SNP in which the minor C allele was invariably methylated. Promoter studies demonstrated reduced activity of the AF007878.1(MMP1):g.3447T>C C allele and evidence for the impact of methylation of the cytosine was obtained through gel shift studies that demonstrated that the methylated C had a higher affinity for a protein in amnion fibroblast NEs that specifically bind to the surrounding DNA sequence. ChIP assays confirmed reduced transcriptional activity of the minor C allele of the -935 SNP. These findings reveal a novel situation where the combination of genetic variation and epigenetic marks affect gene expression.

Our data regarding influences of *MMP1* genotypes on risk of PPROM are pertinent to the understanding of the genetic basis and biological mechanisms underlying this obstetrical complication. Our discovery of the AF007878.1(MMP1):g.3447T>C C allele that reduces *MMP1* promoter function and the previously described AF007878.1(MMP1):g.2775delG 2G allele that increases promoter activity defines promoter haplotypes that must be considered in association studies (4,9). Additional association studies with larger sample sizes are warranted to determine the contributions of specific haplotypes to risk of PPROM and other outcomes in which MMP1 expression could have an influence.

As to the relative roles of genetic and epigenetic variation in the regulation of MMP1 expression, it appears that genetic variation may be the primary determinant, exclusive of the methylation of the AF007878.1(MMP1):g.3447T>C C allele, since significant differences in MMP1 transcription were found when amnion fibroblasts cultures were analyzed based on the AF007878.1(MMP1):g.2775delG and AF007878.1(MMP1):g.3447T>C *MMP1* promoter genotypes independent of any alterations in promoter DNA methylation.

Multiple factors could contribute to risk of PPROM including environmental factors (infection, smoking), abnormalities in amnion extracellular matrix synthesis, and excessive rates of extracellular matrix degradation (1,3,11). MMP1 is but one of a number of enzymes that could participate in extracellular matrix catabolism in the fetal membranes. Other enzymes include MMP2, MMP8 and MMP9 (3). Consequently, our studies probably reflect only a portion of the overall genetic and epigenetic influence over PPROM.

In summary, our observations demonstrate that DNA methylation contributes to the complexity of the control of MMP1 expression. Therefore, we conclude that genetic variation and as well as epigenetic factors should be taken into consideration in association studies.

MATERIALS AND METHODS

Subjects, tissue samples and DNA extraction

Subjects in this study were African-American women and their neonates receiving obstetrical care at the Hospital of the University of Pennsylvania, Philadelphia, PA or Hutzel Women's Hospital of the Detroit Medical Center, Detroit, MI, USA. The samples were collected under protocols approved by the Institutional Review Boards as well as the Institutional Review Board of the National Institute of Child Health and Human Development and written informed consent was obtained from all study participants before sample collection. For the case–control study of the association of the AF007878.1(MMP1):g.3447T>C SNP and PPROM, control samples ($n = 361$) were obtained from neonates of singleton pregnancies delivered at term of mothers with no prior history of PPROM or preterm labor. Cases of PPROM ($n = 284$) were defined as neonates from pregnancies complicated by rupture of membranes before 37 weeks of gestation. The diagnosis of membrane rupture was based on pooling of amniotic fluid in the vagina, amniotic fluid ferning and a positive nitrazine test. These subjects were the same as those studied in our previous analysis of a *SERPINH1* promoter SNP and PPROM (11).

Human placentas were obtained at the time of vaginal delivery or when cesarean section was performed at term. Women with multiple gestations, fetal anomalies, trauma, connective tissue diseases and medical complications of pregnancy requiring induction of labor were excluded. Amniotic membranes were removed from the chorionic membranes, washed with phosphate buffer saline and used for amnion fibroblast preparation (cesarean section specimens only), or stored at -80°C for DNA or RNA extraction. For the study on the relationship of methylation of the -1538 site and PPROM, control specimens ($n = 47$) were derived from

singleton pregnancies delivered at term of mothers without any obstetrical history of preterm birth or PPROM. Cases ($n = 47$) were derived from singleton pregnancies complicated by PPROM.

Genomic DNA used for *MMP1* genotyping was extracted from umbilical cords, cord blood or neonate cheek swabs as previously described (11). Genomic DNA from the amnion for bisulfite sequencing was extracted with the Gentra DNA extraction kit according to the manufacturer's suggested protocol (Gentra, MN, USA).

Bisulfite sequencing

Bisulfite treatment of genomic DNA was performed with the CpGenome^{TM} DNA Modification Kit (Chemicon International Inc., CA, USA) as recommended by the manufacturer. We used an incubation time with sodium bisulfite for 16 h at 50°C because preliminary pilot studies revealed that 20 h incubation resulted in fragmented DNA. We amplified and sequenced the promoter DNA from −1621 to −49. This region contains 14 CpG sites. These regions were amplified by mixing 100 ng of bisulfite-treated DNA with 75 pmol of each primer and 2.5 mM of dNTPs with 0.75 units of Z-*Taq* polymerase (Takara Bio Inc., Shiga, Japan). PCR products of 10 μl were then subjected to the second round PCR with the nested primer sets. The initial denaturation was for 2 min at 94°C, followed by 30 cycles for 2 s at 94°C, 5 s at 58°C and 10 s at 72°C, and final elongation for 7 min at 72°C. Each primer set shown in Table 1 was designed to amplify the top strand. The nested PCR products were separated by 2% of agarose gel electrophoresis, and were extracted with QIAquick Gel Extraction Kit (Qiagen, CA, USA), and then were cloned in the PCR 2.1-TOPO vector (Invitrogen, CA, USA) using TOPO TA cloning kit (Invitrogen). After bacterial amplification of the cloned PCR fragments by standard procedures, eight or more clones from two or three different PCR were subjected to DNA sequencing with ABI Prism 377 (Applied Biosystems, Foster City, CA, USA). The procedure for bisulfite sequencing of plasmid DNA with *MMP1* promoter inserted into the pGL3 vector was the same as above, with the exception that the circular plasmid DNA was first linearized with *Eco*R V followed by bisulfite treatment.

Genotyping of the AF007878.1(MMP1):g.3447T>C single nucleotide polymorphism

PCR products amplified using a forward primer 5′-GGGTGGGGAGTTATCTCATACTC-3′ and a reverse primer 5′-TAGGCAACAGAGAGGGACCCTGTCTAAA-3′ were used for genotyping of the *MMP1* promoter AF007878.1(MMP1):g.3447T>C SNP. After initial denaturation at 94°C for 5 min, PCR was performed for 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by a final 5 min elongation at 72°C. The 124 bp product was digested with the endonuclease Cac8 I (New England BioLabs Inc. MA, USA), which yielded one undigested fragment for the T allele and two fragments of 27 bp and 97 bp for the C allele. The digested fragments were separated on the 10% pre-cast acrylamide gel (Bio-Rad). The AF007878.1(MMP1):g.2775-delG genotype of the *MMP1* promoter was determined as previously reported (4).

Assessment of population stratification

We could not exclude the chance that some of the observed association could be the result of admixture stratification since many African-American populations have substantial admixture that is not evenly distributed throughout the population. To control for admixture stratification, we typed 29 ancestry-informative markers, which are useful for calculating gene flow between West African and Western European populations. These markers were used to calculate the individual biogeographic ancestry levels of the persons in the study in the context of the two primary parental populations using parental allele frequencies and the maximum likelihood as previously reported (12). Our previously published data on the association study between genotype polymorphisms of *MMP8* and *SERPINH1* and PPROM (11,12) demonstrated that there is no significant genetic background difference between PPROM and control samples, which were also used in the case–control study for the AF007878.1(MMP1): g.3447T>C SNP.

Construction of promoter-reporter plasmids

To determine whether the AF007878.1(MMP1):g.3447T>C SNP influences transcription of the *MMP1* gene, we used a previously constructed *MMP1* promoter fragment in the pGL3 vector (4). A mutagenesis kit (Stratagene, CA, USA) was used to create the T and C alleles with a uniform backbone sequence. DNA sequences of the promoter constructs were confirmed before use, and three different plasmid preparations for each construct were tested.

Primary cultures of human amnion fibroblast cells

Minced amniotic membranes derived from the placenta collected at cesarean section were digested with 1 mg/ml collagenase A (Roche Molecular Biochemicals, Indianapolis, IN) in 1× Hanks' balanced salt solution at 37°C for 2 h with shaking. The suspension was passed through a wire mesh filter and then cells were pelleted by centrifugation at 1000 g for 10 min. The cells were washed with 1× Hanks' solution and pelleted again. The pellet was re-suspended in 3 ml of Dulbecco's modified eagle medium (DMEM) and then the re-suspension was separated in a Percoll gradient (5/20/40/60%) by centrifugation at 1500 rpm for 20 min. Amnion fibroblast cells were collected from the inter-media layer, were washed in DMEM, and then were seeded in DMEM containing 10% fetal bovine serum (FBS), penicillin G and streptomycin. The cultured cells were maintained at 37°C in a water-saturated atmosphere under 5% CO_2 in air.

Primary amnion fibroblasts were maintained in culture of DMEM with 10% FBS without antibiotics. For treatment of 5-Aza-2′-deoxycytidine (5-Aza) (Sigma–Aldrich, St Louis, MO, USA), which causes DNA demethylation or hemi-demethylation, 1.2×10^5 cells were seeded into six-well plate in 3 ml of medium. After 24 h of incubation, medium

was removed and cells were incubated in 3 ml of fresh medium containing a final concentration of 10 μM of 5-Aza for 24 h. After treatment, medium was removed and cells were subjected to an additional 24 h incubation in 3 ml of fresh medium without 5-Aza, and then genomic DNA was extracted. In another well, cells were incubated for 24, 48, 72 and 96 h with daily change of medium subsequent to an additional 24 h incubation in 5-Aza-free medium.

For promoter studies, 100×10^5 amnion fibroblasts were plated in individual wells of a 12-well plate. Cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics, IN, USA) with 0.5 μg of the pGL3 vector containing the *MMP1* promoter fragment coupled to the firefly luciferase reporter gene. In each transfection, 25 ng of pRL-TK (Promega, Madison, WI), a control plasmid expressing *Renilla reniformis* luciferase, was used to correct for transfection efficiency. The medium was changed 24 h after transfection, and cultures were continued for an additional 24 h in serum-free medium before collecting cells for the luciferase assays.

Quantitative DNA methylation assay

To determine the rate of methylation at the variable CpG site, quantitative assay was performed according to the previous report with slight modifications. This method was called COBRA. Genomic DNA was treated with sodium bisulfite and the treated DNA was subjected to the first and second PCR as stated above using the same primer sets. To remove the primer dimers and the primer from PCR sample, the first PCR products were purified with High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) during each PCR step. Nested PCR products were digested with *Hpy*CH4 IV (New England BioLabs Inc.), which recognizes the sequence (5′-ACGT-3′) at 37°C after overnight process. *Hpy*CH4 IV can cut −1533 site in 5′-UTR of human *MMP1* when methylated (A*CG*T), but cannot cut when unmethylated (A*TG*T). The PCR product containing −1538 CpG site was 146 bp in length, but the digested product was 115 bp. After digestion, the samples were separated by 4% of NuSieve GTG Agarose (Cambrex Bio Science Rockland Inc., ME, USA) gel electrophoresis. After ethidium bromide staining, gel was photoscanned on a 312 nm transilluminater. Each band was analyzed on a Macintosh computer iMac G5 using the public domain Image J 1.34s program (developed at the U.S. National Institutes of Health). To ensure whether the bisulfite conversion was complete, nested PCR products were digested by *Eco*R V (New England BioLabs Inc.), which recognizes the sequence (5′-GATATC-3′) that should be destroyed by the bisulfite modification. The undigested DNA samples were processed for a quantitative assay. We confirmed in a preliminary study that there was a linear relationship between the photoanalytic data and DNA methylation (data not shown).

Quantitation of matrix metalloproteinase 1 protein

Primary amnion cells were treated with 10 μM or 0.1 μM 5-Aza as noted above. The medium was removed and stored at −20°C until assayed. MMP1 protein in the culture media was quantitated with a Matrix Metalloproteinase-1 Biotrack ELISA System (Amersham Biosciences, Piscotaway, NJ). This ELISA assay kit recognizes the pro- and active forms of MMP1.

Quantitative RT-PCR for matrix metalloproteinase 1 nascent transcripts and mRNA

Total RNA was extracted from amnion cells with TRIzol (Invitrogen) according to the manufacturer's protocol, and stored at −80°C until assayed. Total RNA and mRNA were also extracted from the freshly frozen human amnion tissue using TRIzol Reagent (Invitrogen) and mRNA isolation kit (Dynal, Norway). RNA was DNase-treated with DNA-free reagent (Promega). Total RNA (1–2 μg) was used in 20–40 μl of reverse transcriptase reaction with *MMP1* gene-specific primer 5′-TGCCCACAATTAAAACATCAAAG TT-3′, mRNA using oligo(dT) primer, and Moloney murine leukemia virus reverse transcriptase (Promega). Reverse transcriptase reaction (1 μl, 1:10 diluted) was added into 20 μl of the real-time PCR mix. The cDNA was amplified with SYBR green dye (Applied Biosystems). Reactions were run in triplicate on a Prism 7000 Real-Time PCR machine (Applied Biosystems). To measure nascent MMP1 transcript levels, total RNA was reverse-transcribed using 150 nmol of a MMP1-specific primer (5′-TGCCCACAATTAAAACATCAA AGTT-3′) together with 1.5 nmol of a GAPDH-specific primer (5′-TAGAGGCAGGGATGATGTTCTGGA-3′). Relative fold changes were calculated using the standard curve or the ΔCt method. Primer sets were designed using PRIMER EXPRESS software (Applied Biosystems). Primers for mature mRNAs were designed to span an exon–exon junction, and primers for nascent RNA were designed to be in an adjacent exon and intron. The primer sets used for the real-time PCR of *MMP1* gene were: mRNA forward 5′-GGGCTTGAAGCTGCTTACGA-3′ and mRNA reverse 5′-TGTCCCTGAACAGCCCAGTAC-3′; nascent transcript forward 5′-GATGAAGATGAAAGGTGGACCAA-3′ and nascent transcript reverse 5′-TCAACCAATATTACAATGA AACATGAAG-3′; the internal control GAPDH forward primer 5′-GTATCGTGGAAGGACTCATGACCA-3′ and GAPDH reverse primer 5′-TAGAGGCAGGGATGA TGTTCTGGA-3′.

Luciferase assays

The transfected cells were lysed in lysis buffer and 20 μl aliquots of supernatant were then assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) as previously described (12). Promoter activities were expressed as the ratio between *Photinus* luciferase and *Renilla* luciferase activities.

Electrophoretic mobility shift assay

Nuclear proteins from the primarily cultured human amnion fibroblast cells were extracted as described (12). The following double-stranded oligonucleotide probes (SNP identified in bold captions) were constructed: AF007878.1(MMP1):g.3447T>C T allele sense, 5′-TCATACTCCGCCTG**T**GGATGAGGGGTCTT-3′; T allele

antisense, 5′-AAGACCCCTCATCC**A**CAGGCGGAGTATGA-3′; C allele, 5′-TCATACTCCGCCTG**C**GGATGAGGG GTCTT-3′: C allele antisense, 5′-AAGACCCCTCATCC**G**CA GGCGGAGTATGA-3′; C allele-methylated sense, 5′-TCAT ACTCCGCCTG**C**$^{\mathbf{M}}$GGATGAGGGGTCTT-3′; C allele-methylated antisense was the same oligonucleotide as for the C allele antisense; sense unrelated competitor, 5′-ATGC TGTGAACCTCAGGGTGCTCG-3′; antisense unrelated competitor, 5′-CGAGCACCCTGAGGTTCACAGCAT-3′. The double-stranded synthetic oligonucleotides were labeled with T4 polynucleotide kinase and [γ^{32}P] ATP. The EMSA-binding reaction was mixed in 1X binding buffer (Promega) with 10 μg of nuclear protein and 1×10^5 cpm of ^{32}P-labeled double-stranded oligonucleotide probe (1 ng) with or without unlabeled competitor probe in a total volume of 10 μl. Reaction mixtures were incubated at room temperature for 30 min and then subjected to 8% polyacrylamide gel electrophoresis at 250 V for 4 h. The dried gels were then exposed to X-ray film.

Chromatin immunoprecipitation

Three different primary cultures of human amnion fibroblast cells heterozygous for the AF007878.1(MMP1):g.3447T>C in the *MMP1* promoter and homozygous for the AF007878.1(MMP1):g.2775delG 2G allele were used for the ChIP assays. We used the EZ ChIP Kit (Upstate, USA) and followed the manufacturer's instructions. Cells were lysed in 300–400 μl lysis buffer and sonicated by applying three to four 10 s pulses at power level 4 (Sonic Dismembrator, Model 100). ChIP was conducted with anti-Pol II and anti-IgG. The input control DNA or immunoprecipitated DNA was amplified in a 25 μl reaction volume consisting of: 4 μl eluted DNA template, 300 nM forward primer (5′-GGGTGGGGAGTTATCTCAT ACTC-3′), 300 nM reverse primer (5′-TAGGCAACAGAG AGGGACCCTGTCTAAA-3′), one PCR bead (Amersham Biosciences). PCR runs at 94°C for 5 min, one cycle; 94°C for 30 s; 60°C for 30 s; 72°C for 30 s, 30 or 35 cycles and 72°C for 5 min extension. The 124 bp PCR product was digested by endonuclease Cdc8 I (NBL), which produced 97 bp and 27 bp digested bands for the tested cells with −930 T/C genotype precipitated by anti-Pol. II. The band density was quantitated with a BioRad image analysis system. We calculated the transcription ratio of the T and C alleles by comparing the 124 bp band density versus 1.28 × 97 bp band density as 124/97 = 1.28.

Statistical analysis

Allele frequency comparisons and tests of association were conducted using Pearson Chi-squared and Fisher's exact tests as it is required to account for small sample sizes. Logistic regression was used to estimate OR and 95% binomial confidence intervals. Analyses were done using Stata 8.0 (Stata Corp., College Station, TX, USA). Significant differences in activities among the different promoter constructs are evaluated using the Tukey-Kramer test with $P < 0.05$ considered as significant. Statistical analyses of DNA methylation status were performed with a computer program (Statistical Program for Biosciences v. 9.37). The Chi-squared test was used to evaluate each proportion.

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Conflict of Interest statement. None declared.

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