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Epigenetic Inactivation of the Tissue Inhibitor of Metalloproteinase-2 (*TIMP-2*) Gene in Human Prostate Tumors

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Abstract

Gene silencing via CpG island methylation in the promoter region is one of the mechanisms by which tumor suppressor genes are inactivated in human cancers. Previous studies have shown that the *TIMP-2* gene, which is an endogenous inhibitor of matrix metalloproteinases involved in cell invasion and tumorigenesis, is downregulated or silenced in a variety of human cancer cell lines. Here, we investigated the mechanism underlying TIMP-2 expression in prostate cancer cell lines and primary prostate tumor samples. We observed a strong correlation between promoter hypermethylation and lost expression of *TIMP-2* gene, which was supported by other results demonstrating that promoter demethylation by 5-aza-2′-deoxycytidine and trichostatin A reactivated TIMP-2 and restored its expression in *TIMP-2*-silenced metastatic prostate cell lines. These result were further substantiated by a chromatin immunoprecipitation assay, showing the preferential binding of MeCP2 to methylated CpG island in *TIMP-2*-silenced metastatic prostate cell lines. *In vitro* Matrigel invasion assays showed that reexpression of *TIMP-2* after a combined treatment with 5-aza and TSA in metastatic prostate cells resulted in a significant reduction of tumor cell invasion. Furthermore, CpG methylation of *TIMP-2* promoter was also shown in primary prostate tumors that expressed decreased TIMP-2 protein levels. These results suggest that the downregulation of the *TIMP-2* gene is associated with promoter methylation and that this may play an important role in prostate cancer progression during the invasive and metastatic stages of the disease.

Keywords

CpG methylation; TIMP-2; MeCP2; Prostate cancer

INTRODUCTION

Prostate cancer remains the most commonly diagnosed solid tumor and is the second leading cause of cancer death in men in the United States (Jemal et al., 2003). It is a heterogenous disease, varying from clinically indolent to a rapidly fatal systemic malignancy. Prostate cancer develops to an advanced stage in ~25% of patients (Jemal et al., 2004). Androgen ablation

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remains the primary treatment for advanced prostate cancer, but hormone-refractory prostate cancer recurs invariably within 1 to 2 years (Kasamon and Dawson, 2004). Currently, there is no curative therapy for the advanced or hormone-refractory prostate cancer (Hegeman et al., 2004). Improved understanding of the molecular mechanisms of prostate cancer progression may provide a novel basis for development of effective treatment strategies.

Matrix metalloproteinases (MMPs) are a family of structurally-related proteolytic enzymes that mediate degradation of the extracellular matrix (ECM) and the basement membrane (Liotta et al., 1980; Rao, 2003). High levels of MMP activity have been linked to tumor growth, invasion and tumor-induced angiogenesis (Gokaslan et al., 1998; Yamamoto et al., 1996). Net MMP activity is the result of the balance between the levels of the activated enzyme and tissue inhibitors of metalloproteinases (TIMPs). TIMPs belong to a family of natural inhibitors that control the activity of matrix metalloproteinases (Gomez et al., 1997). Four members of this family have been identified: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. All four TIMPs inhibit active forms of most MMPs, but some differences in their inhibitory properties have been reported (Nagase, 1998). TIMPs are involved in diverse biological processes including cell growth, tumor progression, apoptosis, invasion, metastasis and angiogenesis (Gomez et al., 1997; Valente et al., 1998). TIMP-2 is one of the most frequently investigated members of the group due to its involvement in cancer progression and metastasis (DeClerck et al., 1992; Imren et al., 1996).

Epigenetic inactivation of tumor suppressor genes (TSGs) or tumor-related genes by hypermethylation in the promoter region is a common event in human tumor cell lines and human cancers. Several TSGs are inactivated by this mechanism (Esteller et al., 2001). We have previously demonstrated that the *TIMP-2* gene is expressed in normal human tissues, whereas its expression is downregulated in glioblastomas and metastatic lung tumors (Mohanam et al., 1995). Overexpression of TIMP-2 has been shown to restrict the invasiveness of various tumor cell types *in vitro* (DeClerck et al., 1992; Valente et al., 1998). In addition, TIMP-2 overexpression had inhibitory effects on tumor growth and angiogenesis in a breast cancer mouse model (Hajitou et al., 2001). Taken together, these characteristics strongly suggest an anti-tumor function of the *TIMP-2* gene in cancer progression.

In this study, we investigated the methylation patterns of the *TIMP-2* gene promoter in prostate cell lines and primary prostate tumors. Our results show, for the first time, CpG island methylation in the *TIMP-2* promoter region of a significant percentage of prostate cell lines and tumors which did not express TIMP-2 protein indicating that epigenetic regulation is important in TIMP-2 expression in prostate tumors.

RESULTS

TIMP-2 expression in normal, tumor and metastatic prostate cell lines

Previous studies by our group (Mohanam et al., 1995) and others (DeClerck et al., 1992; Imren et al., 1996; Valente et al., 1998) have established that TIMP expression is downregulated with increasing malignancy and that this downregulation is associated with an imbalance between the production of MMPs and TIMPs and a shift towards a pro-proteolytic state with an invasive phenotype (Fig. 1A). In the present study, we first examined the activities of MMPs (MMP-9 and MMP-2) and TIMPs (TIMP-1 and TIMP-2) in normal, tumor and metastatic prostate cell lines by zymography and reverse zymography, respectively. As a control, we used the fibrosarcoma cell line HT1080, which expresses both TIMPs and MMPs (Yoon et al., 2001). Gelatin zymography revealed high levels of MMP-9 and MMP-2 in the culture medium of metastatic prostate cells (LNCaP, DU145, PC3 and Tramp-C1), whereas it was undetectable in the cell culture medium from normal (RWPE1) and tumorigenic prostate cells (RWPE2) (Fig. 1B, *top*). Notably, reverse zymography showed no MMP-inhibitory activity in the cell

culture medium from metastatic prostate cells, but intense TIMP-1 and TIMP-2 bands were observed in normal prostate cells (Fig. 1B, *bottom*). A similar trend was observed by immunoblot assay as well (Fig. 1C). The tumorigenic RWPE2 cell line showed TIMP-1 inhibitory activity and protein expression, but a barely visible TIMP-2 band was observed at both the activity and protein levels. Thus, TIMP-2 activity and protein levels correlated with their known metastatic potential.

To determine whether these protein expression findings correlated with the biological activity of the prostate cancer cell lines used, we measured the ability of these cells to traverse a matrigel-coated membrane with 8-μM pores, a correlate of metastatic potential *in vivo*. We found that LNCaP, DU145, PC3 and Tramp-C1 cells, which do not express detectable TIMP-2 protein, displayed the invasive potential, whereas RWPE1 cells, which express TIMP-2, exhibited no invasive potential (Fig. 1D).

Downregulation of TIMP-2 expression is associated with promoter methylation in metastatic prostate cancer cell lines

Methylation of CpG dinucleotides in the promoter region is an important mechanism that silences expression of many tumor suppressor genes in cancers (Esteller et al., 2001). Since the *TIMP-2* gene contains a CpG island in its promoter (Fig. 2A), we studied whether methylation of the *TIMP-2* promoter is a possible mechanism for the inactivation of TIMP-2 in metastatic prostate cell lines using methylation-specific PCR (MSP). Only unmethylated and no methylated DNA was detected in RWPE1 cells that expressed a high level of TIMP-2. In contrast, only methylated and no unmethylated DNA was detected in LNCaP, DU145, PC3 and Tramp-C1 cells that do not express TIMP-2 (Fig. 2B). Methylated DNA was more strongly detected than unmethylated DNA in RWPE2 cells that expressed a low level of TIMP-2. These results clearly show that the *TIMP-2* promoter was hypermethylated in all metastatic prostate cell lines that lack TIMP-2 expression.

TIMP-2 re-expression with 5-aza and TSA treatment

To determine the contribution of the *TIMP-2* promoter methylation to the expression of this gene, we treated the promoter-methylated prostate cancer cell lines with varying concentrations of 5-aza and TSA. According to the RT-PCR results, treatment with 20 μM 5-aza for 5 days and 50 nM TSA for an additional 24 h restored an optimal level of TIMP-2 mRNA expression in metastatic prostate cells (Fig. 2C, *top*). These changes in TIMP-2 expression following treatment with 5-aza plus TSA are consistent with the notion that promoter methylation repressed TIMP-2 expression in metastatic prostate cells. To test for an inherent effect of this drug on TIMP-2 expression in cells that normally express TIMP-2, we also examined the effects of 5-aza plus TSA on RWPE1 cells. No significant change in TIMP-2 expression levels was observed in RWPE1 cells after drug treatment (Fig. 2C, *bottom*). To confirm the demethylation effect of 5-aza and TSA, bisulfite sequencing was performed on treated cell lines. The results clearly showed that the promoter was truly demethylated (data not shown). Promoter region CpG dinucleotides were mostly methylated in metastatic prostate cell lines LNCaP, DU145, PC3 and Tramp-C1, which do not express TIMP-2, whereas none of these promoter region CpG islands were hypermethylated in the *TIMP-2*-expressing cell line RWPE1 and 5-aza/TSA-treated cell lines LNCaP, DU145, PC3 and Tramp-C1. These data strongly suggest that hypermethylation was responsible for *TIMP-2* gene silencing.

We next investigated the methyl-CpG-binding proteins (MeCPs) occupancy in the hypermethylated *TIMP-2* promoter in metastatic prostate cells. It is thought that methylated CpG may be bound by MeCPs, which in turn are associated with transcription repression complexes containing mSin3A and histone deacetylase (HDAC) (Yu et al., 2000; Pulukuri and Rao, 2006). Thus, we examined the association of MeCP2 with the *TIMP-2* promoter region

in prostate cell lines using ChIP. We found that MeCP2 were present in the *TIMP-2* promoter region in metastatic prostate cell lines LNCaP, DU145, PC3 and Tramp-C1, but not in *TIMP-2*-expressing normal prostate cell line RWPE1 (Fig. 2D). These results suggest that MeCP2 was associated with the methylated *TIMP-2* promoter, and thus, interferes with transcriptional activity of the methylated promoter.

Effects of 5-aza and TSA treatment on the invasive potential of the metastatic prostate cell lines

To determine the effect of *TIMP-2* promoter methylation on the invasiveness of metastatic prostate cells, the invasive potentials of control and treated DU145 and PC3 cells were determined by matrigel invasion assay. Induction of TIMP-2 expression in DU145 and PC3 cells treated with 5-aza and TSA resulted in a significant reduction of the invasive potential of these cells (Fig. 3A). Representative pictures are shown in Figure 3B. Incubation of these cells with the anti-TIMP-2 antibody blocked this acquired reduction in invasive potential (Fig. 3A). This provided further convincing evidence that this decrease in tumor cell invasion after treatment with 5-aza and TSA is due to the induction of TIMP-2 expression.

Downregulation of TIMP-2 in human prostate cancer

Since our data are consistent with a potential tumor-suppressive function for TIMP-2, we assessed if TIMP-2 was disturbed in human prostate cancer. To address this question, TIMP-2 expression was evaluated in the human prostate tissue using immunohistochemistry. TIMP-2 expression was classified as negative, weak and strong in 17 (41.2%), 21 (48.6%) and 4 (10.2%) tumor tissue samples, respectively (Fig. 4A). There was a significantly lower level of TIMP-2 protein expression in the tumors than in the normal tissue samples ($p<0.01$, Fig. 4A) and the representative pictures were presented in Figure 4B. To further confirm these observations, immunoblot analysis was performed using four-paired human normal prostate and tumor tissue samples with known levels of TIMP-2 (Fig. 4C). It was clear that the tumor tissue samples exhibited a downregulation of TIMP-2 protein expression as compared with the normal prostate tissue, which was consistent with the level of TIMP-2 protein expression determined by immunohistochemical staining. We also examined mRNA expression within twenty-paired human normal prostate and tumor tissue samples by real-time PCR analysis (Fig. 4D). Consistent with the results of the immunohistochemistry and immunoblotting, TIMP-2 mRNA was significantly downregulated in the majority of tumor samples when compared with their adjacent normal tissue (Fig. 4D).

Methylation status of TIMP-2 promoter in human prostate tumors

To determine whether TIMP-2 inactivation by DNA methylation is characteristic of human prostate tumors, we performed MSP analysis with primer sets targeting the sequence around the most frequently methylated sites (Fig. 2A) in a panel of 42 human prostate tumors, 32 normal prostate and 15 BPH tissue samples. Only methylated and no unmethylated DNA was detected in 25 of 42 human prostate tumors (Fig. 5A). Methylated DNA was more strongly detected than unmethylated DNA in the eight other prostate tumors. In contrast, only unmethylated DNA was detected in 27 of 32 normal prostate samples (Fig. 5B). Partial methylation was detected in 4 of 15 BPH samples, with unmethylated DNA being more strongly detected than methylated DNA in one of these cases and the other ten BPH samples exhibited only unmethylated DNA (Fig. 5C). The methylation profile of the *TIMP-2* promoter in normal, BPH and prostate tumors is summarized in Figure 5D. We further analyzed the methylation status of the *TIMP-2* promoter using bisulfite sequencing (Fig. 6). Nearly all 25 CpG sites within the CpG island of TIMP-2 were methylated in prostate tumors. In contrast, CpG sites of *TIMP-2* promoter were either unmethylated or sparsely methylated in normal (Fig. 6A) and tumor-matched adjacent normal tissues (Fig. 6B). The prevalence of *TIMP-2*

promoter methylation in prostate tumors is strongly consistent with the lack of TIMP-2 expression seen in the majority of prostate tumors, whereas the lower frequency of promoter methylation in BPH and normal prostate samples is consistent with the presence of TIMP-2 expression in these tissues (Figs. 4–6).

DISCUSSION

Our studies show that methylated TIMP-2 can be detected in metastatic prostate cell lines and TIMP-2 mRNA expression can be reestablished after demethylation with 5-aza and TSA (Fig. 2). After treatment with 5-aza and TSA, an induction of TIMP-2 mRNA expression in DU145 and PC3 cells resulted in a considerable reduction of the invasive capacity of these cells. Specificity of these TIMP-2 mediated effects was shown by incubating these experimental DU145 and PC3 cells with an anti-TIMP-2 antibody that significantly reversed this acquired reduction in tumor cell invasive capacity. These results provided convincing evidence that induction of TIMP-2 expression is indeed responsible for the lower invasive capacity of these cells (Fig. 3). Our observations are in line with the previously shown inhibitory effect of TIMP-2 on the invasive ability of fibrosarcoma HT1080 tumor cells (Yoon et al., 2001).

Our studies using clinical prostate samples have shown that majority of the prostate cancer tissues ($n = 42$) have weak or no expression of TIMP-2 when compared with BPH or normal prostate tissues (Fig. 4). Additionally, we have shown that the downregulation of TIMP-2 in prostate cancer is due to epigenetic control of its expression via gene promoter region hypermethylation (Figs. 5 & 6). These findings provide further insight into earlier observations that TIMP-2 was differentially expressed in a variety of human malignant tumors (Mohanam et al., 1995).

Previous studies on other members of the TIMP family including TIMP-3 suggest that methylation is important for the regulation of TIMP-3 expression (Bachman et al., 1999; Pennie et al., 1999). Recent data identified downregulation of TIMP-2 expression by epigenetic mechanisms (Galm et al., 2005; Ivanova et al., 2004; Suzuki et al., 2002). Suzuki *et al*. (2002) observed methylated TIMP-2 in the colorectal cancer cell line RKO. However, methylation of TIMP-2 was not commonly found in primary colorectal tumors. Ivanova *et al*. (2004) used MSP and sodium bisulfite analysis of genomic DNA from expressing and unexpressing cervical cancer cell lines to show a correlation between gene activity and demethylation of the 5′region of the *TIMP-2* gene. Galm *et al*. (2005) recently showed that treatment of lymphoma cell line Raji and the leukemia cell line KG1a with 5-aza induced *TIMP-2* gene expression. These two recent studies established the frequency of hypermethylated *TIMP-2* genes in both lymphoma (38.6%) and cervical cancer (47%). In the present study, methylation of the *TIMP-2* promoter was demonstrated in primary prostate samples at a high frequency (78.5%). We believe that genetic variability between the different cancers may account for the differences. Consistent with this notion, the methylation profile of individual genes seems to be different in various tumor types. Certain genes, such as *14-3-3σ*, *RASSF1A* and *P16INK4a*, are commonly methylated in multiple human cancers (Wajed et al., 2001; Pulukuri and Rao, 2006), whereas others show high frequencies of methylation only in specific tumors. One example is the *GSTP1* gene that is highly methylated in breast and prostate cancers but is largely unmethylated in other types of tumors (Das and Singal, 2004; Esteller et al., 2001).

In summary, the present study shows that hypermethylation of the *TIMP-2* gene correlates with the silencing of TIMP-2 expression in prostate cancer cell lines and in primary prostate tumors. Further studies of methylation profiles of a large number of primary tumor samples will provide important insight into the role of CpG island hypermethylation of the *TIMP-2* promoter in tumor progression.

MATERIALS & METHODS

Cell lines, 5-aza-2′-deoxycytidine and trichostatin A treatment

The prostate cancer cell lines RWPE1, RWPE2, LNCaP, DU145, PC3 and Tramp-C1 were obtained from the American Type Culture Collection and cultured as directed. Total RNA and genomic DNA were isolated from the treated cells using RNA and DNA isolation kits. Cells were treated with 5-aza and TSA as previously described (Pulukuri and Rao, 2006).

Zymography

Confluent prostate cells were maintained in serum-free media for 24 h. Equal amounts of concentrated media were loaded on to 10% polyacrylamide gels containing 2.5 mg/mL of gelatin (Sigma, St. Louis, MO). After electrophoresis, the gels were washed and stained as described previously (Yamamoto et al., 1996). The substrate degrading enzymes MMP-9 and MMP-2 were identified as clear bands in the blue background. For reverse zymography, concentrated media was electrophoresed in 15% polyacrylamide reverse zymograms containing 2.5 mg/mL gelatin and 0.15 μg/mL MMP-2 (Oliver et al., 1997). TIMP activity was visualized as dark zones of gelatinase inhibition against a pale, partially digested background.

Human prostate tissues and immunohistochemistry

Paired human prostate tumor and normal adjacent tissues were obtained from patients undergoing routine therapeutic surgery. Many prostate cancer, BPH and normal tissues were also obtained as paraffin-embedded, formalin-fixed blocks. For IHC, tissue sections were labelled with mouse monoclonal anti-human TIMP-2 antibody (Oncogene, San Diego, CA) and graded the expression levels as negative, weak positive and strong positive (see Supplementary section).

Methylation-specific PCR

We analyzed prostate cancer, BPHs and normal prostate tissue samples. DNA was extracted and denatured with NaOH and treated with sodium bisulfite for 16 h. We used the following MSP primers: unmethylated TIMP-2 sense: 5′- TTT GGT GTT TTG GAA GAA TGG GTG-3′ and antisense 5′- CCA ACC CCA ATC CCC ACT ACA-3′; methylated TIMP-2 sense: 5′- TTT GGT GTT TTG GAA GAA CGG GCG-3′ and antisense 5′- CGA CCC CGA TCC CCG CTA CG-3′. Positive controls used for methylation-specific PCR included DNA from RWPE1 cells as the unmethylated DNA control and CpGenome Universal methylated DNA as the methylated DNA control (Chemicon, Temecula, CA). Negative control MS-PCR reactions were performed using water only as template.

Cloning and bisulfite sequencing

To permit DNA sequencing of inclusive CpG sites within the CpG island of the *TIMP-2* gene, sodium bisulfite-treated DNA was amplified with primers common to methylated and unmethylated DNA sequences. Amplified PCR products were purified with a Gel extraction kit (Qiagen, Valencia, CA) and ligated into pCR2.1-TOPO plasmid vector with a TA-cloning system (Invitrogen, Carlsbad, CA). Plasmid-transformed *E. coli* were cultured and plasmid DNA was isolated with FastPlasmid mini-prep kit (Eppendorf, Westbury, NY). Purified plasmid DNA containing the TIMP-2 sequence was sequenced with an automated DNA sequencer. Bisulfite sequencing was performed in 10 prostate cancer samples, 10 matched non-neoplastic adjacent tissues and 10 normal prostate samples. Samples that had clones with >50% methylation of CpGs were designated partially methylated. Samples containing clones with >75% methylation of CpGs sites were designated methylation-positive. All other samples were designated methylation-negative.

Immunoblot, ChIP and RT-PCR analysis

Immunoblotting, ChIP and RT-PCR were performed as described previously (Pulukuri and Rao, 2006). Expression analysis for TIMP-2 mRNA was measured using real-time quantitative PCR with SYBR Green PCR Mastermix (Bio-Rad, Hercules, CA). (see Supplementary section).

Matrigel invasion assay

Invasion of cells through matrigel was conducted using a Transwell apparatus (Corning Costar) as described previously (Yoon et al., 2001). (see Supplementary section).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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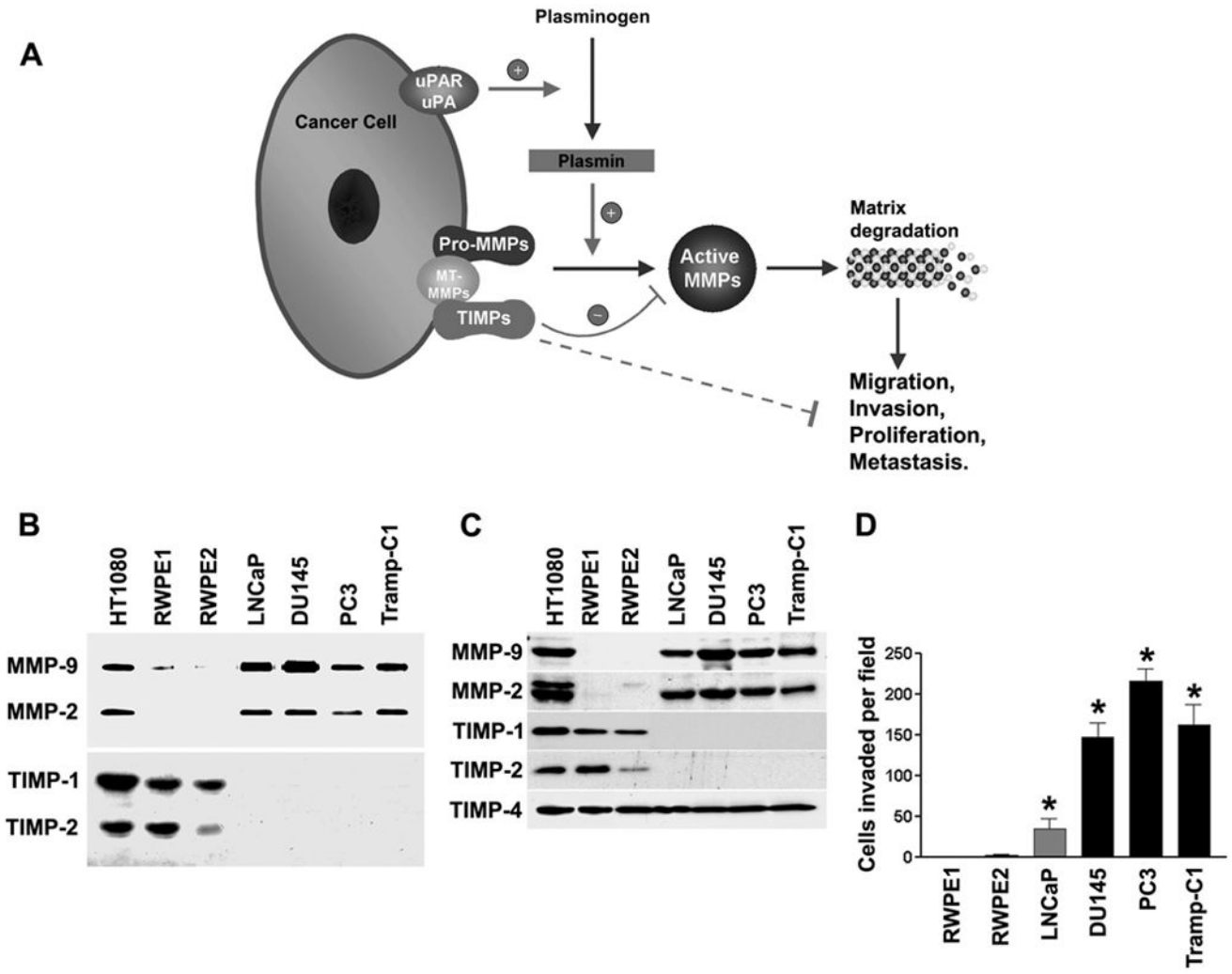


Figure 1. Activity and expression levels of TIMP and MMP proteins in prostate cell lines

(A) A model for the balance between TIMP and MMP activity and the subsequent effects on tumor cell invasion.

(B) MMP (*top*) and TIMP (*bottom*) activity levels in prostate cell lines RWPE1, RWPE2, LNCaP, DU145, PC3 and Tramp-C1 were assessed by zymography and reverse zymography, respectively.

(C) Immunoblot analysis of TIMP and MMP proteins in the concentrated culture medium of prostate cell lines. TIMP-4 was used as a loading control.

(D) Cells invading through the matrigel were counted under a microscope in three random fields at 200X magnification. Each bar represents the mean ± SD of three fields counted where significant differences from normal prostate cancer cell lines RWPE1 are represented by asterisks (*) ($p<0.01$).

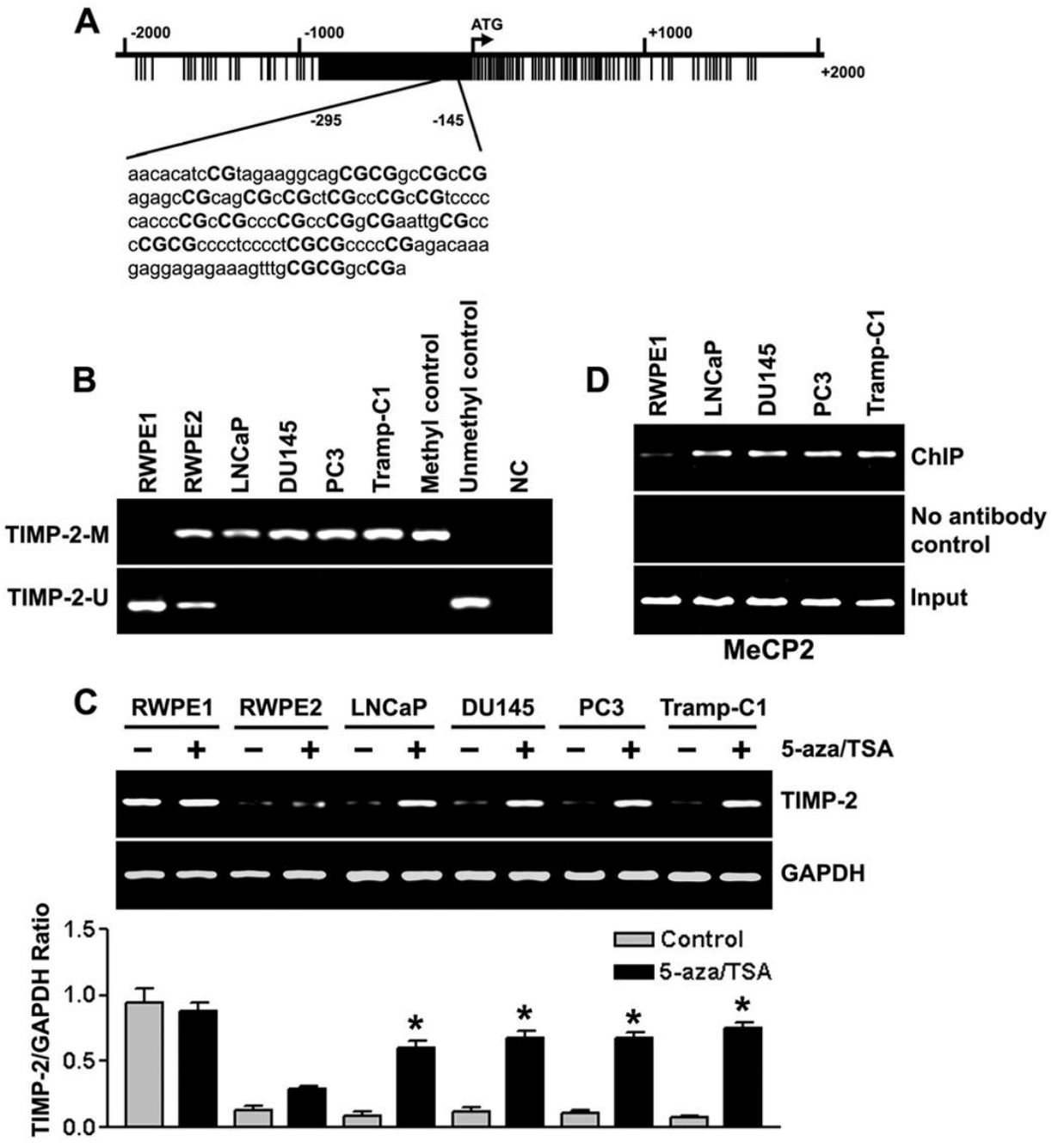


Figure 2. Analysis of *TIMP-2* promoter CpG methylation in prostate cancer cell lines

(A) Schematic diagram indicating the CpG island on *TIMP-2* promoter region. Nucleotide sequence located in the promoter region of the human *TIMP-2* gene from −295 to −145 with respect to the translation initiation codon (bent arrow) is shown. CpG dinucleotides are indicated in bold.

(B) Bisulfite-modified DNA derived from prostate cancer cell lines were amplified with TIMP-2 primers specific for unmethylated and methylated DNA. TIMP-2-U, unmethylated PCR product; TIMP-2-M, methylated PCR product; NC, no template control. Positive and negative controls were described in Materials & Methods.

(C) mRNA expression of TIMP-2 was analyzed using RT-PCR in prostate cancer cells treated with 20 μM 5-aza-CdR followed by 50 nM TSA, as described in Materials & Methods. GAPDH mRNA was amplified as a loading control and expression standard. Bar diagram showing densitometry quantified data of TIMP-2 mRNA/GAPDH mRNA ratios from three independent experiments. Asterisks (*) indicate significant differences from untreated control cells ($p<0.01$).

(D) Assessment of MeCP2 binding on the *TIMP-2* promoter by ChIP analysis. The bound MeCPs fraction (ChIP) shows binding to the *TIMP-2* promoter. Aliquots of chromatin taken before immunoprecipitation were used as “input” controls whereas chromatin eluted from immunoprecipitations lacking antibody were used as “no antibody” (-Ab) controls.

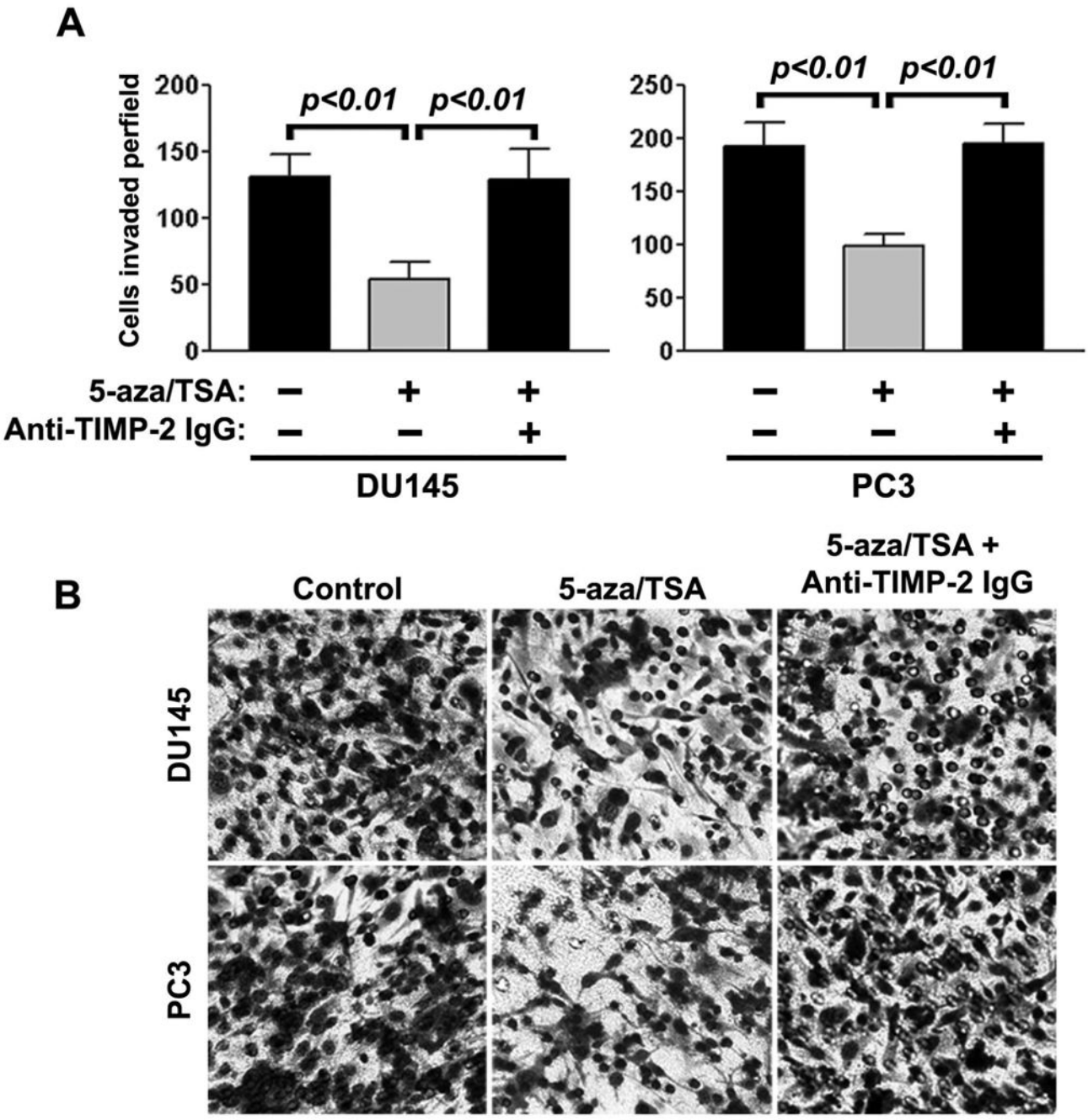


Figure 3. Effects of *TIMP-2* promoter CpG methylation on cellular invasion in DU145 and PC3 cells

(A) The invasive capacity of the untreated (Control) and 5-aza-treated DU145 and PC3 cells were assessed *in vitro* by matrigel invasion assay. Results are the mean ± SD of three different experiments. Significant difference from control is represented by asterisks ($p<0.01$).

(B) Representative invasive potentials of the untreated (Control) and 5-aza-treated DU145 and PC3 cells by matrigel invasion assay.

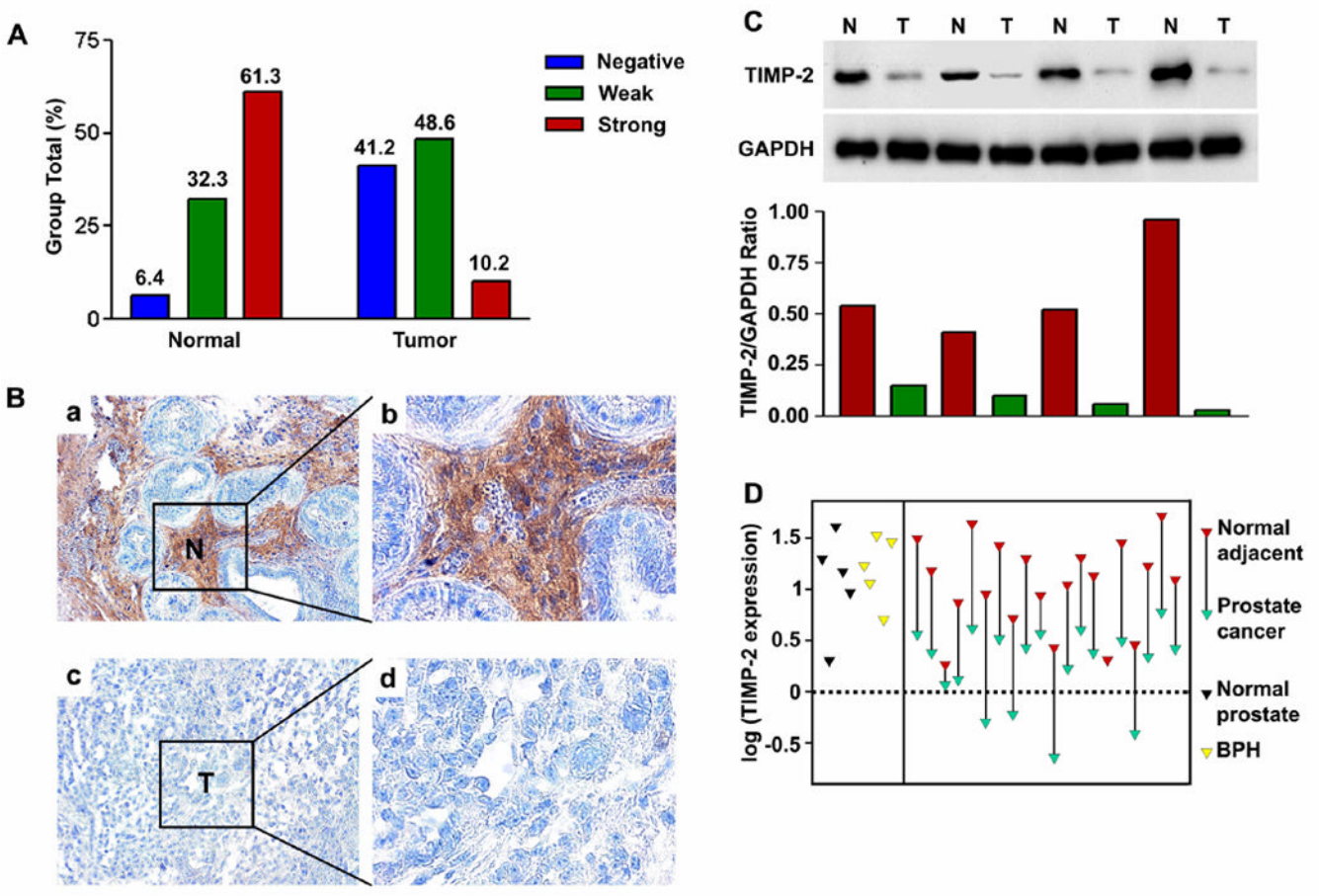


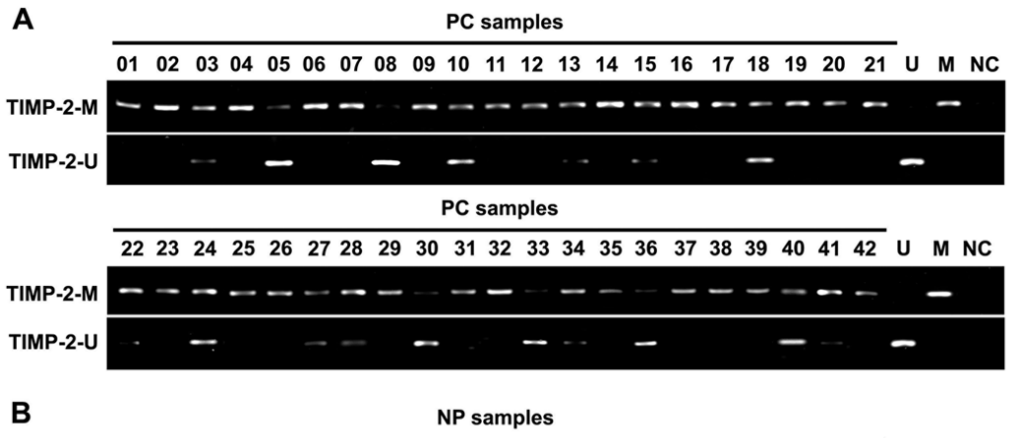
Figure 4. Expression of TIMP-2 in human prostate tissue samples

(A) Compared with normal prostate tissues, the overall expression level of TIMP-2 in prostate cancer tissues was significantly lower ($p<0.01$). TIMP-2 expression by normal human prostate tissue (32 cases) and prostate cancer (42 cases) was analyzed.

(B) Representative immunostaining photographs were taken at different magnifications: *a*, normal human prostate showing TIMP-2 in epithelial cells; *b*, high-power view of (*a*) showing membrane staining of TIMP-2; *c*, lack of TIMP-2 staining in prostate cancer tissues; *d*, high-power view of (*c*) showing lack of TIMP-2 expression in prostate cancer tissues ($p<0.01$); *T*, tumor cells; *N*, normal prostate epithelial cells.

(C) Total cell extracts were further prepared from four-paired normal prostate (*N*) and prostate tumor tissue (*T*) specimens. The levels of TIMP-2 protein expression were determined by immunoblot analysis. The level of TIMP-2 protein expression was significantly lower in tumor tissue than in normal tissue, which was indicated by the ratio of TIMP-2/GAPDH (*bottom*).

(D) Quantitative mRNA expression of TIMP-2 in human prostate tumor (green) compared to normal adjacent tissue of the same individuals (red, connected by a line). Significant downregulation of TIMP-2 expression in tumor tissue than in normal adjacent tissue samples.



D Summary of methylation results in prostate tissues.

Tissue	Methylation status		*P*-value
	Unmethylated	Methylated	
Normal vs tumor (*n* = 89)			
BPH	11/15 (73.3%)	4/15 (26.6%)	
Normal	27/32 (84.3%)	5/32 (15.6%)	
Tumor	9/42 (21.4%)	33/42 (78.5%)	<0.001

Figure 5. Methylation status of *TIMP-2* promoter in normal and prostate cancer tissue samples
MSP analysis was performed on **(A)** prostate cancer **(B)** normal prostate and **(C)** BPH tissue samples. **(D)** Summary of TIMP-2 methylation results in formalin-fixed prostate tissue consisting of 42 prostate cancers, 15 BPH samples and 32 normal human prostate tissues.

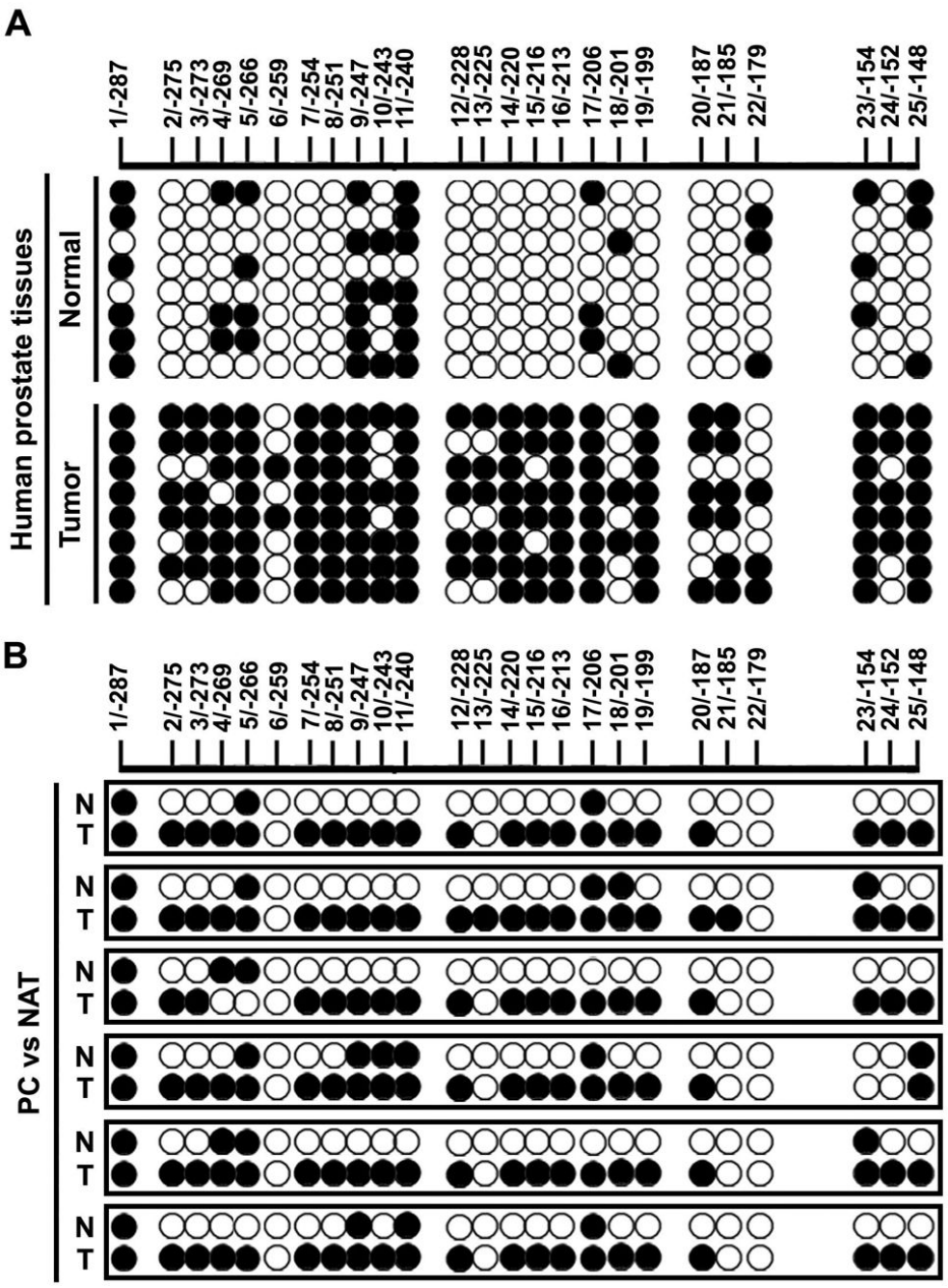


Figure 6. Methylation patterns of the CpG island of *TIMP-2* in human prostate tissues

CpG positions are indicated relative to the translation start codon; each circle in the figure represents a single CpG site. Representative sequencing results of the MSP products. A filled circle represents a methylated CG dinucleotide, and an empty circle represents a demethylated CG dinucleotide.

(A)DNAs of ten normal prostate and prostate tumor samples that displayed methylated *TIMP-2* in tumor (*T*) and unmethylated *TIMP-2* in normal prostate tissue (*N*) was selected for genomic sequencing; the selected prostate tumor samples were negative in TIMP-2 protein expression.

(B) DNAs of ten normal adjacent tissue (NAT) samples that displayed unmethylated TIMP-2 in assays of MSP and their tumor counterparts displayed decreased TIMP-2 expression in real-time PCR analysis were selected for genomic sequencing.