Aberrant expression of the *PRAC* gene in prostate cancer

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Abstract. Identification of aberrant expression patterns of genes in prostate cancer (PCa) is a key step towards the development of effective therapies. Prostate-specific antigen (PSA) levels are commonly measured for the early detection of PCa, but which itself is still not an ideal biomarker. We analysed the expression patterns of prostate cancer susceptibility candidate (*PRAC*) in prostate cancer. The *PRAC* gene is known to be commonly expressed in prostate tissue, rectum and colon. To provide clear insights into the expression patterns of *PRAC* in PCa, we examined the gene expression by quantitative real-time PCR (qRT-PCR), western blot analysis and immunohistochemistry (IHC). The results showed that *PRAC* expression levels in androgen-insensitive cells (DU145 and PC3) are lower than those in androgen-sensitive cell lines (LNCaP, LNCaP-R and CW22R). However, treatment of the LNCaP cell line with androgen and anti-androgen demonstrated that *PRAC* is expressed in an androgen-independent manner. Further, PRAC expression was restored upon treatment of DU145 and PC3 cells with the methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-CdR), which indicates the effect of methylation in the control of *PRAC* expression. In addition, IHC analysis revealed a significantly decreased immunoreactivity of PRAC protein in PCa tissues compared to benign prostatic hyperplasia (BPH) ($p<0.0001$). Thus, our findings suggest that the pathogenesis of PCa may be due to the expression levels of PRAC protein, and this protein can serve as a potential biomarker for the management of PCa.

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Introduction

Prostate cancer is a heterogeneous disease due to which the search for genetic causes involved in the pathogenesis remains a challenge. Deregulated expression of several genes such as *EZR* and *OCT1* were identified, and the involvement of the aberrant expression of these genes was reported in the pathogenesis of PCa (1,2). The variety in biological behaviour of PCa demands identification of biomarkers that may distinguish a slow growing cancer from a more aggressive cancer with a potential to metastasize (3).

The androgen receptor is a ligand-dependent zinc finger DNA-binding protein that is involved in the regulation of transcription of a variety of gene derivatives (4). The unique feature of PCa is its dependency of androgen for its growth and survival. Several novel androgen-regulated genes have been identified, some of which may be important in the regulation of prostate cell invasiveness (5). In general, androgens activate the androgen receptors which in turn control the expression of androgen receptor response elements (ARE) containing genes due to which current research targets androgen-based therapies for PCa.

Epigenetic factors are also known to mediate the expression of several genes. DNA methylation is one of the epigenetic mechanisms (6) and it occurs in mammals mostly at cytosines within CpG dinucleotide. Several studies have been proposed that DNA hypomethylation can cause activation of oncogenes and genetic instability, whilst hypermethylation is associated with inappropriate gene silencing (7). For instance, Lin *et al* reported the role of hypermethylation in the silence of glutathione-S-transferase P1 (*GSTP1*) expression in PCa (8). It is reported that *GSTP1* is hypermethylated in nearly all human prostate cancers and its promoter DNA methylation level is able to differentiate between BPH and different grades of prostate adenocarcinoma (9-11).

PRAC is a novel gene encoding for the 382 nucleotide RNA, and it specifically expressed in prostate tissue, rectum and colon. The sequence tag database is a potential source for discovery of new genes (12,13), and it was used to find the *PRAC* gene (14). The *PRAC* gene is located on chromosome 17 at position 17q21, 4 kbp downstream from the homeodomain *Hoxb-13* gene. To date, there is no specific study on the prognostic role and regulatory factors that govern the expression of

Table I. PRAC immunohistochemical score from benign and cancer prostate tissues.

Clinical samples	Immunostaining			P-value
	Strong	Moderate	Weak	
Tissues				
BPH (n=27)	21 (77.7%)	6 (22.2%)	0	<0.0001
PCa (n=27)	2 (7.4%)	7 (25.9%)	18 (66.7%)	
Pathological GS				
≤3+4 (n=7)	2 (28.5%)	3 (42.8%)	2 (28.5%)	
≥4+3 (n=20)	0	4 (20%)	16 (80%)	

PRAC gene. In this study, we have identified the distinct difference in the expression patterns of PRAC protein between PCa and BPH tissues. Additionally, regulatory role of methylation in the expression of *PRAC* gene was demonstrated.

Materials and methods

Cell lines and clinical tissues. In total, five PCa cell lines including DU145, PC3, LNCaP, LNCaP-R and CWR22R were obtained from the American Type Culture Collection (Manassas VA, USA). DU145 and PC3 are known as aggressive and androgen insensitive cell lines whereas LNCaP, LNCaP and CWR22R are relatively less aggressive and androgen sensitive cells. Immunohistochemical analysis was carried for the prostate specimen of 54 patients with equal number of BPH and PCa tissues were represented (Table I). All the clinical samples were approved by the Research Ethics Committee of Chang Gung Memorial Hospital (Tao-Yuan, Taiwan) with the approval no. 95-0345B.

Cell culture. The cells were cultured in RPMI-1640 containing 10% FBS, 50 mg/ml each of penicillin and streptomycin and the medium was replaced every alternative day. To synchronize the cell cycle, all prostate cells used in this study were incubated in RPMI media without serum for 24 h. These cancer cell lines were further used to analyse the expression of PRAC gene both at mRNA and protein levels.

RNA extraction and qRT-PCR. Total RNA from the cultured PCa cell lines were extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) using the protocol recommended by the manufacturers. Total RNA was quantified and analysed by spectrophotometry (NanoDrop Technology Inc., Wilmington, DE, USA). The cDNA was prepared by using SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen). qRT-PCR reactions were performed using SYBR-Green SuperMix (Bio-Rad, Hercules, CA, USA) in 20 μl total volume and a Bio-Rad iCycler iQ Real-Time Detection System according to the manufacturer's instructions. Primers for target genes including *PRAC* (forward, 5'-GCCCATTTCTCAGATCA AGG-3'; reverse, 5'-GGTCTCGCCCAGTAGATGTT-3'), *PSA* (forward, 5'-AGGTCAGCCACAGCTTCCCA-3'; reverse, 5'-GGGCAGGTCCATGACCTTCA-3'), *GSTP1* (forward, 5'-CAATACCATCCTGCGTCACCT-3'; reverse, 5'-GCAAG ACCTTCATTGTGGGAG-3') and *β-actin* (forward, 5'-CATG TACGTTGCTATCCAGGC-3'; reverse, 5'-ATCGTGCGTGA CATTAAGGAG-3') were designed using Primer 3 online tool (15). PCR reactions were performed in triplicate, and relative expression level of target genes in all the cell lines was calculated by normalizing to *β-actin* expression levels using the comparative threshold cycle (CT) method. CT represents the cycle numbers at which the amplification reaches a threshold level chosen to lie in the exponential phase of all PCR reactions. Data were analysed using the iCycle iQ system software (Bio-Rad).

Protein extraction and immunoblot assay of human PRAC, AR and β-actin. Cultured cells were lysed in Pro-Prep™ Protein Extraction Solution (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Proteins were analysed by spectrophotometry (NanoDrop Technology Inc.). For western blot analysis, cell lysates were separated by using 4-20% Tris-glycine precast gel (Bio-Rad) and transfered to 0.2-μm Immobilon-PSQ PVDF membrane (Millipore, Billerica, MA, USA). Later, membrane was blocked with 5% non-fat milk in TBS-T buffer (150 mM NaCl, 10 mM Tris/pH 8.0 and 0.05% Tween-20) at room temperature for 1 h or overnight at 4˚C. Then, the membranes were immunoblotted with diluted PRAC primary antibodies (1:500) (Abnova, Walnut, CA, USA) for 1 h at room temperature or overnight at 4˚C, followed by incubation with secondary antibodies (AP124P, Chemicon, Millipore) for 1 h at room temperature. Blots were visualized by a chemiluminescence ECL system (Millipore).

Immunohistochemistry. IHC analysis was performed after approval from institutional review board. PCa and BPH tissues embedded in paraffin were cut into 5-mm sections. The sections were dewaxed in xylene and rehydrated in ethanol (Sigma Chemical Co., St. Louis, MO, USA). For antigen retrieval, paraffin sections were boiled for 20 min in 10 mM sodium citrate buffer. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck, Hohenbrunn, Germany) for 10 min and tissue sections were then incubated with 1% bovine serum albumin (Invitrogen) for 30 min. The sections were then incubated with the diluted PRAC primary antibody (1:200) (Abnova). After washing in TBS containing 0.1% Tween-20, the sections were then further incubated with SuperPicture HRP Polymer conjugate antibody (Zymed/Invitrogen, Carlsbad, CA, USA) for 30 min. The peroxidase reaction was visualised using a liquid DAB

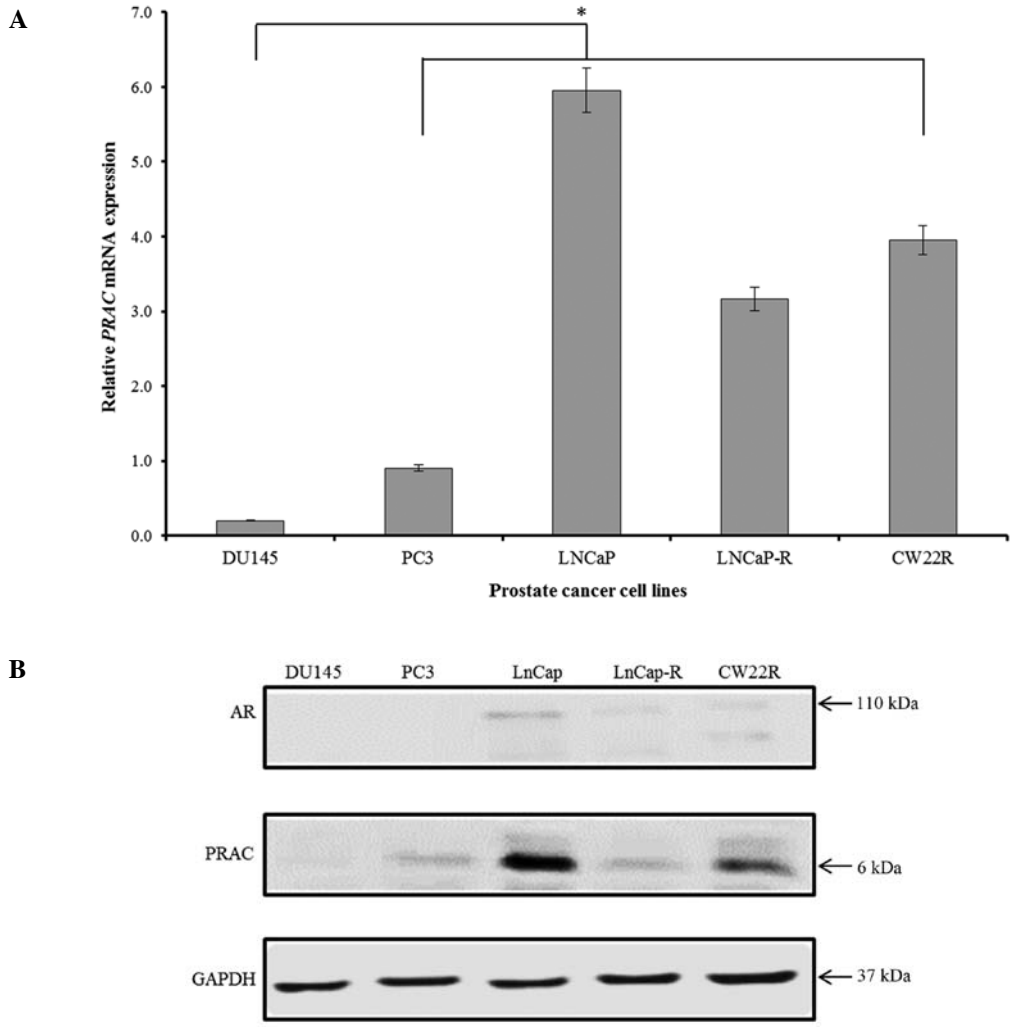

Figure 1. Gene expression of *PRAC* in various prostate carcinoma cells. (A) Transcriptional level expression of *PRAC* gene in different cell lines was determined by qRT-PCR. *p<0.05, significant difference of *PRAC* expression between androgen insensitive (DU145 and PC3) and androgen sensitive (LNCaP, LNCaP-R and CW22R) cells. (B) Translational level expression of *PRAC* gene in different cell lines was determined by western blot assay and the PRAC protein levels were correlated with the levels of AR protein.

substrate kit (Dako, Carpinteria, CA, USA). All sections were counterstained with hematoxylin (Dako) for 20 sec. Finally, the sections were microscopically observed. Immunostaining of PRAC was scored independently. IHC stains were scored: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining; based on their intensity. In addition, IHC stains were graded on a semi-quantitative scale according to the prevalence of nuclear fraction positivity within the tumor cells (0, <10%; 1+, 10-25%; 2+, 25-50%; 3+, 50-75%; and 4+, >75%). Nuclear positivity of PRAC protein was scored by multiplying the percentage of positive cells (P) by the intensity (I).

Hormonal treatment. LNCaP cells were seeded at low density (about 1x10^6 cells) in 100-mm cell culture plates. The medium was then substituted with phenol red-free RPMI-1640 with 5% Dextran coated charcoal-treated FBS (HyClone Inc., Logan, UT, USA) in order to avoid any interfering factor that might modify the metabolic ability of the cells. The cells were preconditioned for 24 h in medium containing two different flasks prior to exposure to 1 nM DHT and 1 μM flutamide (Sigma Chemical Co.). In one flask, 1 μM of flutamide was added into the medium 1 h before the DHT inclusion. Control flasks received vehicle (medium with ethanol only). Following 24 h of incubation in androgen containing medium, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and harvested by scraping and transferred to sterile plastic tubes, and stored at -80˚C until analysis. Cells from the control flasks were harvested in the same way.

5-aza-CdR treatment. DU145 and PC3 cells were seeded at low density (about 1-1.5x10^6 cells) in 100-mm cell culture plates. After 24 h, the cell lines were treated with different final concentrations of 5-aza-CdR (Sigma Chemical Co.) at 1, 5 and 10 μM. Since the downregulation of *PRAC* was identified in well known aggressive cell lines including DU145 and PC3, restoration of *PRAC* mRNA was analysed after 4 days of treatment with 5-aza-CdR. *GSTP1* expression was simultaneously analysed as positive control.

Statistical analysis. The data, representative of three or more independent experiments are presented as the mean ± SEM. To reveal the statistical significance, unpaired t-test was carried

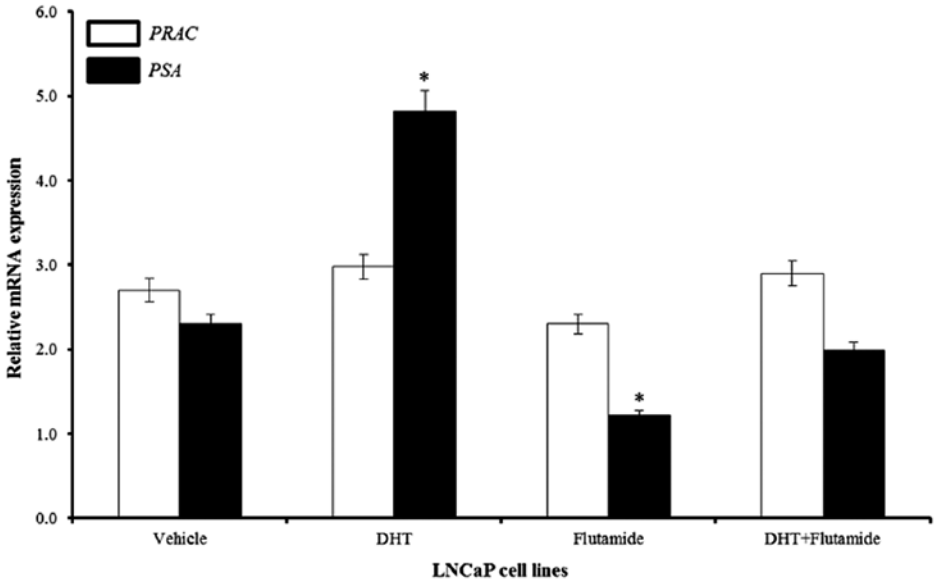

Figure 2. Action of androgens on *PRAC* gene expression. LNCaP cells were treated with vehicle, 1 nM DHT, 1 μM flutamide or 1 nM DHT plus 1 μM flutamide for 24 h. The cells were harvested and total RNA was prepared and converted to cDNA for qRT-PCR analysis of *PRAC* and *β-actin*. *p<0.05, significance of *PSA* (positive control) expression.

out using GraphPad software in which p-value <0.05 was considered as significant.

Results

Expression analysis of PRAC in PCa cell lines. Initially, *PRAC* expression was screened in five PCa cell lines including DU145, PC3, LNCaP, LNCaP-R and CW22R. The results of qRT-PCR and immunoblot assays showed the high expression level of *PRAC* in all the androgen sensitive cells including LNCaP, LNCaP-R and CW22R, whereas its expression was significantly downregulated (p<0.05) in the androgen insensitive cell lines DU145 and PC3 (Fig. 1). Moreover, immunoblot assay revealed a strong association of PRAC protein levels with the levels of AR protein (Fig. 1B). These results initiate a speculation that PRAC might be regulated by androgens.

Evaluation of the androgen effect on PRAC expression. Since higher *PRAC* levels were identified in androgen sensitive cell lines, we treated LNCaP cell lines with the various concentrations of DHT. Our quantitative expression analysis showed slight changes in the expression of *PRAC* after the DHT treatment. To further clarify this difference in *PRAC* expression, we treated the LNCaP cell line with flutamide, an anti-androgen. However, there was no significant change in the expression of *PRAC* after the treatment with flutamide, whereas the well known androgen regulated candidate *PSA*, showed significant response to the androgen treatment (p<0.05) (Fig. 2). Our results indicate that androgens do not have significant role in the regulation of *PRAC* expression, and thus the *PRAC* is expressed in an androgen-independent manner.

The PRAC gene contains a CpG island near the transcription start site. Analysis of the *PRAC* gene (near the transcription start site) using MethPrimer CpG Island finder (16) revealed the presence of two CpG islands. The first island totals 6 CpG pairs and spans a region of 110 bp (+27 to +136). This CpG island covers the region downstream of the transcription start site, the first exon (which is translatable), and part of the first intron adjacent to exon 1. The second island totals 9 CpG pairs and spans a region of 142 bp (+213 to + 354) and covers a part of intron 1 (Fig. 3A). This identification led us to further study the regulatory role of methylation on *PRAC* expression.

PRAC expression analysis in methyltransferase inhibitor treated DU145 and PC3 cell lines. We treated DU145 and PC3 cell lines with the 5-aza-CdR to identify the effect of methylation in the control of *PRAC* expression. Interestingly, significant increment in the expression of *PRAC* was found when the DU145 and PC3 cell lines were treated with higher concentration (10 μM) compared to lower concentrations of 5-aza-CdR treated and wild-type cell lines (p<0.05). *GSTP1* expression was simultaneously analysed as positive control, and the distinct difference in the expression of *GSTP1* was also identified (p<0.05) (Fig. 3B). These results provide initial evidence for the role of methylation in the regulation of PRAC expression.

IHC analysis revealed aberrant expression of PRAC in prostate cancer. IHC analysis was carried to evaluate the correlation of the PRAC immunoreactivity in BPH and PCa tissues (Fig. 4A). We noted that there is an apparent trend in the reduction of PRAC expression in aggressive cancer. BPH tissues from most patients (77.7%) frequently showed profound nuclear immunostaining, whereas a majority of cancer tissues (66.7%) showed weak immunoreaction which is supporting the downregulation of PRAC protein in cancer tissue. Additionally, most of cancer patients (80%) with high Gleason scores (≥4+3) showed weak nuclear positivity of PRAC protein compared to cancer tissues with low Gleason

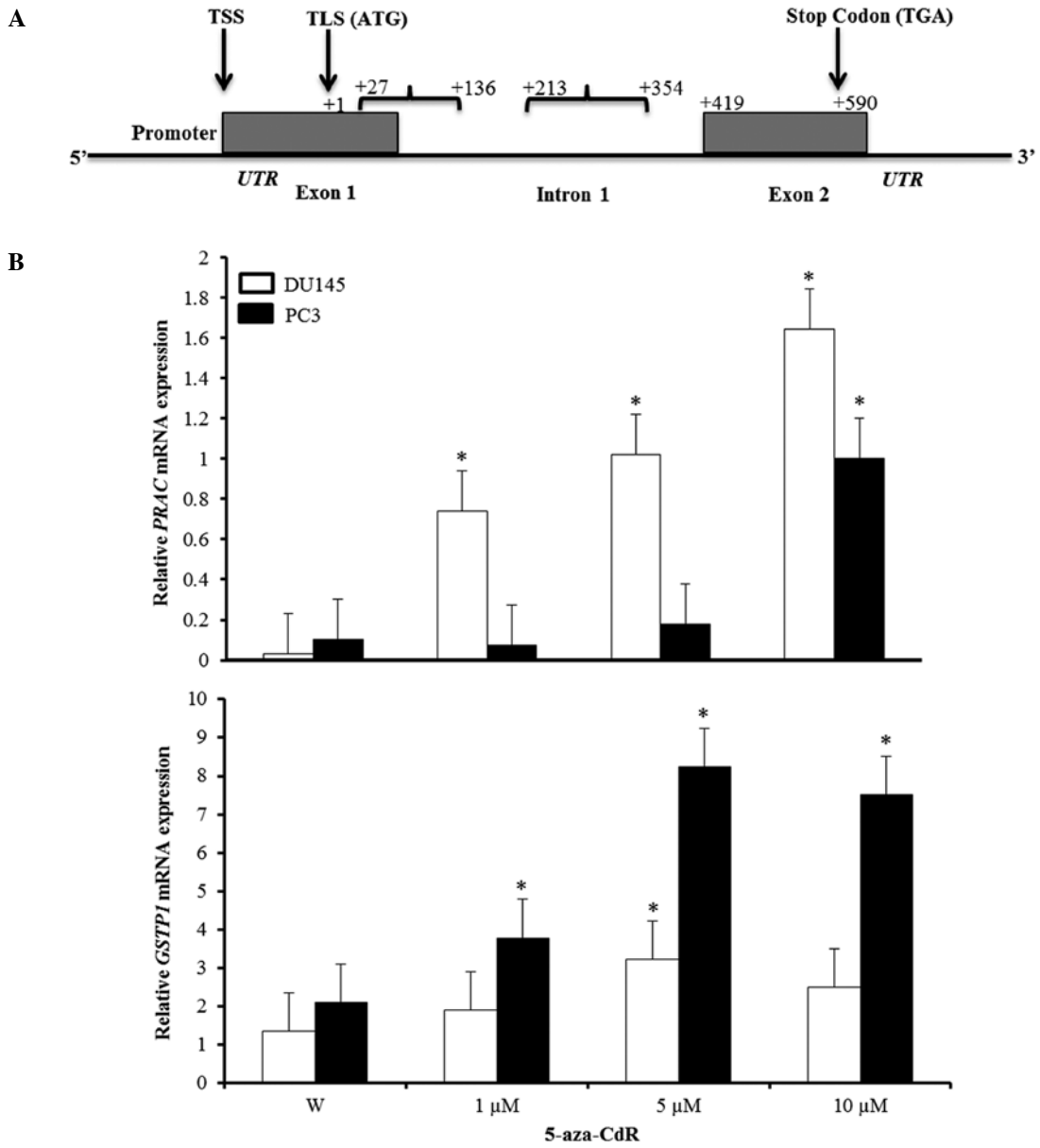

Figure 3. Analysis of methylation effect on *PRAC* gene expression. (A) The schematic structure of human *PRAC* gene and 2 CpG island(s) were found near the translational start site as indicated in the location of CpG island [island 1, 110 bp (+27 to +136) and island 2, 142 bp (+213 to + 354)]. Nucleotide positions are numbered relative to the TLS. TSS, transcriptional start site; TLS, translational start site; UTR, untranslated region. (B) Reversal of *PRAC* silencing by 5-aza-CdR. Levels of *PRAC* mRNA before (W, wild-type) and after the treatment with 5-aza-CdR (1, 5 and 10 μM). Levels of mRNA were measured by qRT-PCR (normalized to β-*actin* mRNA). *GSTP1* was used as a positive control. *p<0.05, the significance of the expression of *PRAC* and *GSTP1*.

score (≤3+4) (Table I). The difference between PRAC staining intensity in cancer and benign samples was found to be significant (P<0.0001) (Fig. 4B).

Discussion

Clinical therapeutic effectiveness of PCa has been challenged by significant cellular heterogeneity and limited understanding of the genetic elements governing disease progression (17). Although, it was found that *PRAC* is expressed specifically in prostate, rectum and colon, there were no studies on the importance of the *PRAC* gene in the PCa pathogenesis and regulatory factors that govern the expression of the gene. Therefore, we analysed its expression patterns in human benign and malignant prostate tissues. Initial expression analysis demonstrated the expression of PRAC protein is possibly related to the AR expression (Fig. 1B) which delineates that androgen receptor (AR) may have an essential role in the regulation of *PRAC* expression. It is well known that AR plays a central role in regulating the growth of the PCa cells (18). Therefore, research has been focusing on the role of AR regulated genes during PCa progression. Several previous findings indicated that the expression of the *PSA* gene in LNCaP cells is regulated by androgens (19,20). Due to its tissue specificity and androgen inducibility, the *PSA* gene has been used as a reference gene to study androgen action in PCa. In the present study, we treated the LNCaP cell line (endogenous AR containing line)

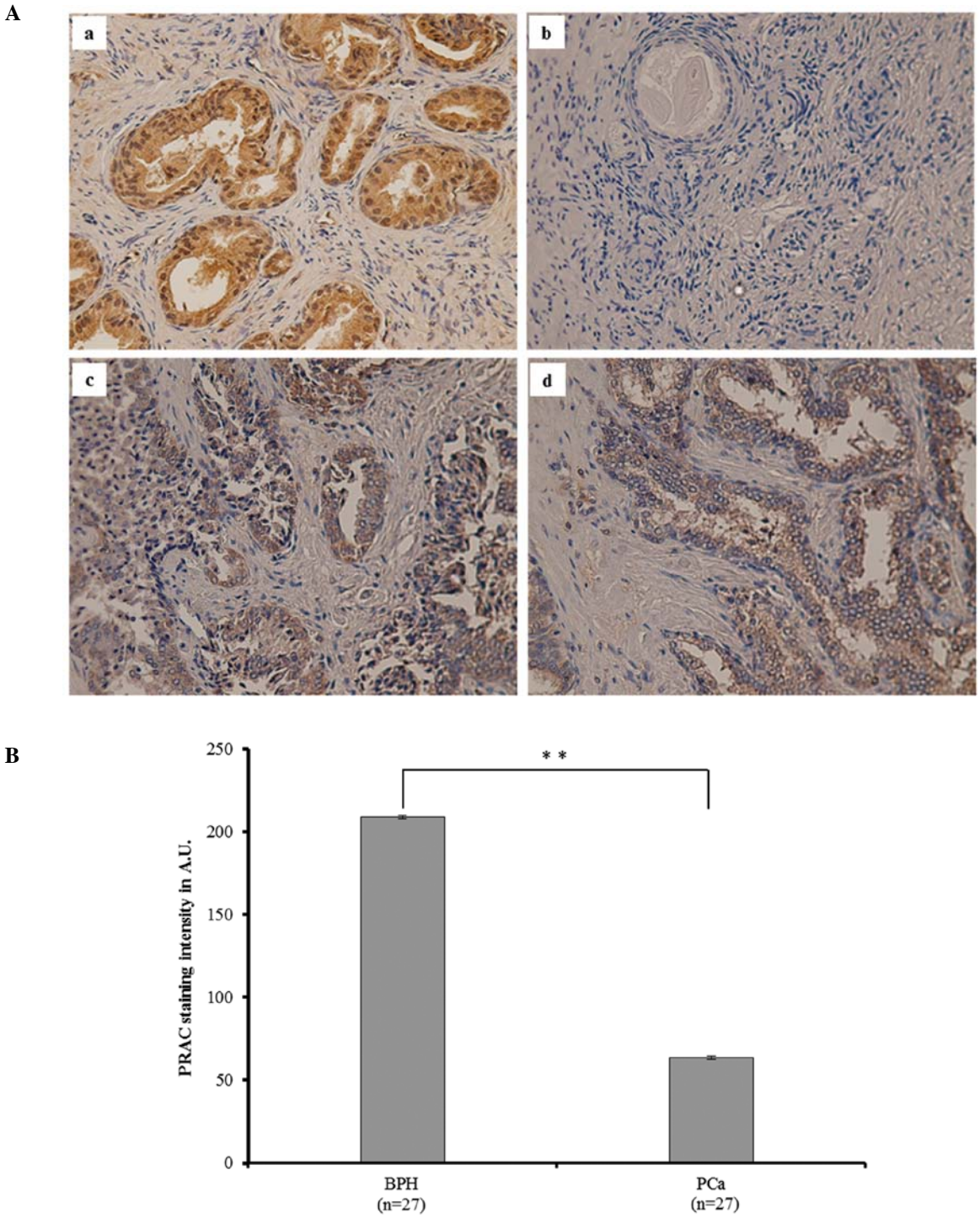

Figure 4. Immunohistochemistry of PRAC protein in BPH and PCa specimens. (A) Panel (a) indicates PRAC protein expression in the BPH tissues. As shown in panel (b), BPH tissues were also used as negative control (NC) in which the tissues were incubated without primary antibody. These tissues specimens were used to compare the PRAC immunoreactivity with PCa tissues which are shown in panels (c) and (d). (B) The mean values of the IHC quantification in arbitrary units (A.U.) are shown. **p<0.0001, the significant difference in PRAC immunoreactivity between PCa and BPH tissues. n=27, the number of patients included in the study.

with DHT and flutamide. Contrary to expectation, *PRAC* was found to be regulated in an androgen-independent manner (Fig. 2). *PRAC* is located at chromosome band 17q21 immediately adjacent to the *PRAC2* and *Hoxb-13* genes, which have also been proposed as markers of human PCa (14,21). In addition, *Hoxb-13* was reported to be expressed only in the prostate and colon of mice in an androgen-independent manner, which is consistent with our expression studies of *PRAC* in LNCaP cell lines (22). This increases the possibility that *PRAC* and *Hoxb-13* are under the same transcriptional control.

Studies have proven that epigenetic alterations are common events in cancer including PCa, which may lead to aberrant expression of critical genes such as tumor suppres-

sors and oncogenes (23,24). Moreover, it was found that various epigenetic inhibitors such as DNA methyltransferase inhibitors, 5-aza-cytidine (5-aza-CR or Vidaza) and its more potent analogue 5-aza-CdR or decitabine can chemically reverse the expression of genes altered due to the epigenetic alterations (23,24). As it was well established that these inhibitors effectively restore the expression of *GSTP1* in PCa cells, herein to investigate the efficacy of 5-aza-CdR in PCa cells, we used *GSTP1* as reference gene (25). Interestingly, significant increment in the expression of *PRAC* was found when the DU145 and PC3 cell lines were treated with higher concentration of (10 μM) compared to lower concentrations of 5-aza-CdR treated cell line and wild-type cell line ($p<0.05$). To further support the efficacy of 5-aza-CdR in PCa cells, the distinct difference in the expression of *GSTP1* was identified ($p<0.05$) in DU145 and PC3 cells (Fig. 3B). These findings indicate that understanding the molecular mechanism for the methylation of the *PRAC* gene may provide insights into the development of PCa.

To our knowledge, only one study with a few samples has demonstrated expression pattern of *PRAC* in PCa (14). Moreover, it has been identified that location of *PRAC* gene has been shown to undergo loss of heterozygosity (LOH) in PCa (26,27). If the *PRAC* genes were located within the LOH region, there could possibly be a reduction in *PRAC*/*PRAC2* expression in PCa. These findings urged us to analyse the expression patterns of PRAC protein in cancerous versus benign prostate tissue using a larger cohort of well-defined PCa patients. Interestingly, PRAC protein was found to be downregulated in cancerous tissue as compared to BPH ($p<0.0001$) (Fig. 4B). These results highlight the possible correlation between *PRAC* expression and invasiveness of PCa. Moreover, we are the first to demonstrate the epigenetic factor, methylation, effects on *PRAC* gene expression in prostate carcinoma cells, and also androgen-independent regulation of *PRAC* expression. The decreased expression of PRAC protein could be due to the effect of methylation, which needs to be further studied. In conclusion, these results suggest that PRAC protein may play an important role in the pathogenesis and probably can be a biomarker for PCa.

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