

# Phosphodiesterase sequence variants may predispose to prostate cancer

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## Abstract

We hypothesized that mutations that inactivate phosphodiesterase (PDE) activity and lead to increased cAMP and cyclic guanosine monophosphate levels may be associated with prostate cancer (PCa). We sequenced the entire PDE coding sequences in the DNA of 16 biopsy samples from PCa patients. Novel mutations were confirmed in the somatic or germline state by Sanger sequencing. Data were then compared to the 1000 Genome Project. PDE, CREB and pCREB protein expression was also studied in all samples, in both normal and abnormal tissue, by immunofluorescence. We identified three previously described PDE sequence variants that were significantly more frequent in PCa. Four novel sequence variations, one each in the *PDE4B*, *PDE6C*, *PDE7B* and *PDE10A* genes, respectively, were also found in the PCa samples. Interestingly, *PDE10A* and *PDE4B* novel variants that were present in 19 and 6% of the patients were found in the tumor tissue only. In patients carrying PDE defects, there was pCREB accumulation ( $P < 0.001$ ), and an increase of the pCREB:CREB ratio (patients  $0.97 \pm 0.03$ ; controls  $0.52 \pm 0.03$ ;  $P$ -value  $< 0.001$ ) by immunohistochemical analysis. We conclude that PDE sequence variants may play a role in the predisposition and/or progression to PCa at the germline and/or somatic state respectively.

## Key Words

- ▶ prostate cancer
- ▶ PCa
- ▶ phosphodiesterases
- ▶ PDE family
- ▶ CREB
- ▶ pCREB
- ▶ cAMP
- ▶ cGMP

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## Introduction

Phosphodiesterases (PDEs) are grouped into 21 families based on their structural homology and biochemical features (Azevedo *et al.* 2014). They function as enzymes that regulate intracellular levels of cAMP and cyclic

guanosine monophosphate (cGMP) which in turn affect the function of a multitude of molecules (Halpin 2008). Recently, protein–protein interactions have been described for several of the PDEs (Jensen *et al.* 2009).

These data add to the complexity of PDEs in modulating multiple signaling pathways, such as those of the protein kinases A, B and C (PKA, PKB, PKC respectively) and others (Hoffmann *et al.* 1998, Baillie *et al.* 2000, MacKenzie *et al.* 2000, Shakur *et al.* 2001, Zhao *et al.* 2002, Pozuelo Rubio *et al.* 2005). PKA, in particular, is essential for PDE function both for mediating cAMP effects, and through A-kinase anchoring proteins (AKAPs), which bind and localize PDEs in the cell (Pan *et al.* 2012). PDE4 is the most thoroughly studied PDE in terms of interactions with other molecules, such as arrestins and receptors for activated C kinase 1 (RACK1; Pan *et al.* 2012).

Pharmacological exploitation of PDEs has led to the discovery of drugs with selective action against specific isoforms. Selective PDE inhibitors, such as sildenafil, are widely used for the treatment of conditions ranging from erectile dysfunction to heart failure and pulmonary hypertension (Tsertsvadze *et al.* 2009). Other PDE inhibitors are considered for therapeutic treatment in diseases such as asthma and chronic obstructive pulmonary disease (Kodimuthali *et al.* 2008). PDE5A inhibitors (PDE5i) relax smooth muscle cells by increasing intracellular levels of Ca<sup>2+</sup>, and raise cGMP levels through activation of the K<sup>+</sup> channels in endothelial cells (Luedders *et al.* 2006). PDE5is are used to treat benign prostatic hyperplasia (Hotston *et al.* 2006, Wong *et al.* 2009, Wang 2010) and it has been shown *in vitro* that they induce apoptosis and control cell proliferation (Cook & Haynes 2004, Zhu *et al.* 2005, Tinsley *et al.* 2009). A recent study suggests that the effects of the PDE5i Zaprinate (Sigma-Aldrich) may control cell proliferation in human cultured prostatic stromal cells, in a time-of-exposure- and dose-dependent way (Cook & Haynes 2004).

Even though some studies have shown that increased cGMP through PDE5A inhibition controls cell proliferation, other studies have produced contradictory data. PDE5is are also capable of inhibiting PDE6, which may play an important role in cell cycle arrest (Cote 2004, Wang *et al.* 2004, Ma & Wang 2007, Bazhin *et al.* 2010). tadalafil, a drug that is widely used for erectile dysfunction is, in addition to its PDE5i role, the strongest PDE11A inhibitor (Washington & Shindel 2010). PDE11A-inactivating mutations and sequence have been linked to predisposition to prostate cancer (PCa), testicular germ cell cancer, and adrenal tumors (Horvath *et al.* 2006a,b,c, 2009, Faucz *et al.* 2011, Libe *et al.* 2011).

In this study, we sequenced the PDE exome in PCa samples. Our data show higher frequency of PDE sequence variants as compared to the 1000 genomes database. Furthermore, patients with PDE sequence variants had upregulated cAMP signaling in their PCa tissues.

## Materials and methods

### Patients and DNA preparation

We collected frozen tumors tissues and blood from 48 males of white, mixed European descent. The Biobank of AC Camargo Cancer Center, Brazil provided the samples (Campos *et al.* 2012, Olivieri *et al.* 2014). Patients' stage of disease, age, PSA and Gleason scores can be found in Supplementary Table 1, see section on supplementary data given at the end of this article. Written informed consent was obtained from all participants, and the institutional review boards of the participating centers approved the study. Biopsy samples were obtained at the moment of PCa diagnosis. Peripheral DNA was extracted from blood samples; tumor tissue DNA was extracted from the frozen biopsy samples from all patients following standard procedures. DNA from 16 (out of the 48) biopsy tissues was subjected to targeted sequencing using Agilent's SureSelect SOLiD4 platform (Life Technologies). Germline DNA from peripheral blood was used to confirm the origin of the novel mutations. Selective variants identified in the group of 16 were further studied in the remaining 32 PCa patients by Sanger sequencing.

### Library capture and sequencing

For targeted sequencing, we selected 203 genes that included all the known PDEs, and molecules related directly and through networks to cAMP and cGMP signaling. Genomic DNA (3 µg) from each of the 16 affected tissues was used for sequencing library preparation. Target enrichment was performed using the Agilent's SureSelect solution-based capture assay (Gnirke *et al.* 2009); libraries were prepared following the manufacturer's protocols (SureSelect Target Enrichment System for the Applied Biosystems SOLiD System). Paired-end sequencing (50 nt read length) was performed on the SOLiD4 platform (Life Technologies), according to the manufacturer protocol.

### Data analysis

Briefly, high quality trimmed reads were mapped and aligned to the human reference genome (Hg 18) using Biosoftware version 1.3 (SOLiD BioScope Software, Life Technologies, Carlsbad, CA, USA). The variants were called using GATK (Broad Institute Cambridge, MA, USA), and annotated through SeattleSeq v5.07 (Ng *et al.* 2009). All variants considered for further study were visually

examined using the Integrative Genomics Viewer (IGV) (Robinson *et al.* 2011).

### Sanger sequencing

To validate novel variants, we performed Sanger sequencing using a 3130xl Genetic Analyzer (Applied Biosystems). To verify the origin of the novel variations (not previously reported), Sanger sequencing was performed on both somatic and peripheral DNA. Furthermore, *PDE5A* variants were studied in all 48 PCa patients. Primer sequences and PCR conditions are available upon request.

### Statistics

The *P*-value and odds ratio (OR) was used to determine significant differences between the frequencies of single-nucleotide variants (SNVs) among the patients and 379 European individuals from the 1000 Genome Project (EUR) and all 1092 individuals from the 1000 Genome Project (ALL). Both European and general population data were collected from the 1000 Genome Project (Genomes Project *et al.* 2010), which was extracted through the Ensembl Genome Browser ([www.ensembl.org/index.html](http://www.ensembl.org/index.html)). To determine significant differences among groups, a two-way analysis was performed, with 95% confidence and calculated through Fisher's exact test and Bonferroni correction using the IBM SPSS software 20 (Endicott, NY, USA). SNVs with  $P < 0.05$  when analyzed individually and lost its statistical power after the Bonferroni correction were kept separately in a new table since our sample size is small and their statistical significance might change with a larger sample size. Immunohistochemical (IHC) statistical analysis was performed through *t*-test with similar parameters used for Fisher. Furthermore, we performed the Mann–Whitney–Wilcoxon test, which is a nonparametric test of the null hypothesis that two populations are the same against an alternative hypothesis that patients tend to have a greater amount of variations than controls, with significance value of  $P < 0.1$ . Both *t*-test and Mann–Whitney–Wilcoxon were performed through RStudio (v.0.98; [www.rstudio.com](http://www.rstudio.com)) and R (v.3.1.1; [www.r-project.org](http://www.r-project.org)).

### IHC analysis

IHC was performed on three patients with high number of PDE variants, two patients with low number of PDE variants and on three age-matched control tissues obtained from other surgeries and/or necropsies. For this experiment, three areas of each slide were used for comparison of

each control and each patient's with high number of PDE variants' tissue; expression was graded for each reading (nine for the patients with high number of PDE variants and nine for the controls) without knowledge of the origin of the tissue. Tissues from only two patients with low number of PDE variants were available. Therefore they were not included for the statistical analysis.

We studied the expression of cAMP-responsive element binding protein (CREB) and its phosphorylated status (pCREB). Slides from PCas were deparaffinized and rehydrated through a series of alcohol solutions (100, 95, 70 and 50% and 1XPBS). Followed by heat-induced antigen retrieval, whereby slides were boiled in Antigen Unmasking Solution (Citric Acid Based, pH 6.0; Vector Laboratories, Burlingame, CA, USA), for 30 min in a standard rice cooker. All slides were blocked with 10% normal goat serum (NGS, JacksonImmunoResearch, West Grove, PA, USA) made in 1XPBS for 1 h at room temperature and then sections were incubated with the following primary antibodies: rabbit anti-CREB and mouse anti-pCREB (both from Cell Signaling, Danvers, MA, USA; dilution 1:100) overnight at 4 °C. The next day, slides were washed in 1XPBS, 3 × 15 min at room temperature. All slides were then incubated with Alexa Fluor Goat anti-rabbit 488 and Alexa Fluor Goat anti-mouse 555 (Life Technologies) for 1–2 h. Again, slides were washed in 1XPBS, 3 × 15 min at room temperature. Cover slips were affixed to each slide with ProLong Gold Antifade Reagent with DAPI (Life Technologies). For negative control, a section was incubated under identical conditions with no primary antibody. Fluorescence was analyzed with a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) and images acquired using the Olympus DMP7 camera using the CellSens image acquisition software. For each channel, separate images were acquired and all images were collected at the same exposure. Following image acquisition, fluorescent staining for pCREB and CREB was quantified blindly, using ImageJ imaging software (Schneider *et al.* 2012). By delimitating the area, the mean fluorescent amplitude was determined from the integrated pixel intensity for the whole area on both the 488 and 555 nm emission channels separately.

To determine the level of CREB phosphorylation, a ratio of the mean fluorescent amplitude measured by the excitation of Alexa Fluor 555 bound to pCREB was divided by the mean fluorescent amplitude measured by the excitation of Alexa Fluor 488 bound to CREB for all slides. Relative pCREB:CREB ratios were reported for each area and for each group as mean ± s.e.m. Differences were tested for statistical significance by using the *t*-test and the level of significance was set at  $P < 0.05$ .

## Results

### DNA sequencing

Analysis of the sequencing datasets revealed an average of 184 kb covered region per sample. We identified 106 known SNVs and 4 novel variants within the PDE gene family among the 16 randomly selected biopsies DNA. Distribution of the variants was assessed between our group and the general population, taking into account the ethnicity (Supplementary Table 2). Because our patients were of mixed European–Caucasian descent, only those control individuals (EUR) were considered for comparison.

From these 106 SNVs, three were significantly more frequent among PCa patients as compared to the controls ( $P < 0.05/106$ , after correction for multiple trials) (Table 1); for the remaining 19 there was no statistical significance when analyzed within the group (Table 2). Most of these known SNVs are absent or extremely rare ( $\leq 1\%$ ) in the EUR, with the exception of rs3733526, which has minor allele frequency (MAF  $< 20.1\%$ ). Among these SNVs, six were significantly overrepresented in PCa patients compared to both ALL and EUR populations.

To determine the somatic or germline nature of the novel PDE variants, we performed Sanger sequencing in both tumor and blood-derived DNA. This analysis identified two variants that were present in the tumor tissue only. One was a novel non-synonymous (ns) substitution in the *PDE4B* (Table 3). The second was a novel *PDE10A* intronic variant located near the splice site of the exon 5 (c.347-9 A>C) identified in 3 out of 16 samples (Table 3). *In silico* analysis of potential splice alterations resulting from this variant was performed using five different web-based tools: SplicePort (Dogan et al. 2007), ASSP (Wang & Marin 2006), Sroogle

(Schwartz et al. 2008), NetGene2 (Hebsgaard et al. 1996) and BDGP (Reese et al. 1997). They all predicted a significant change in the splice acceptor. Furthermore, bioinformatics analysis of this variant using the MutationTaster software (Schwarz et al. 2014) consider this variation as disease causing (probability: 0.9999) due to increased chances of splice site shifting and evolutionary conservation. The remaining two novel variants were a nsSNV in *PDE7B* (p. Met239Ile) and an intronic variant in *PDE6C* (Table 3). Even though the *PDE7B* variation was near the splice site, no significant change in the splicing regions was predicted through *in silico* modeling. All the previously reported variants were originally described in studies of germline DNA. Therefore, we did not anticipate seeing them in the somatic setting, as we did in our study.

Among the 106 known SNVs, 19 were nsSNVs. Each nsSNV ( $n = 21$ ; including the two novel ones) was counted in each individual from both control ( $n = 379$ ,  $\bar{x} = 2.59$ , s.e. = 0.07) and patient ( $n = 16$ ,  $\bar{x} = 3.5$ , s.e. = 0.58) groups. The average number of PDE nsSNV in patients was 35% higher than in the general EUR population with a significant  $P$ -value of 0.07996 ( $< 0.1$ ; Mann–Whitney–Wilcoxon test; Fig. 1 and Table 4).

### IHC analysis

Patients carrying high number of PDE variants had a highly significant increase of pCREB ( $P$ -value  $< 0.001$ ), whereas, CREB levels seemed to be approximately the same between controls and patients (a representative sample from one patient with high number of PDE variants, one patient with low number of PDE variants and one control is shown in Fig. 2). Thus, the pCREB:CREB ratio (pCREB:CREB) was significantly higher in patients ( $0.97 \pm 0.03$ ) vs controls ( $0.52 \pm 0.03$ ;  $P$ -value  $< 0.001$ ) (Table 5, Fig. 3).

**Table 1** Three significant associated SNVs with prostate cancer

Gene	SNV	Location/distance to splice	SIFT/PPH2	Population	Alelic $P$ -value/OR (CI)
<i>PDE2A</i>	rs577536	c.2112+3 A>G 3 bp	*	EUR	$P = 6.1 \times 10^{-5}$ OR = 1.10 (95% CI = 0.98–1.23)
			*	ALL	$P = 0.028$ OR = 0.19 (95% CI = 0.05–0.67)
<i>PDE11A</i>	rs75461311	Intron > 10 bp	*	EUR	$P = 1.4 \times 10^{-4}$ OR = 26.9 (95% CI = 6.40–113.0)
			*	ALL	$P = 0.029$ OR = 3.80 (95% CI = 1.30–11.1)
<i>PDE11A</i>	rs79903863	p.Glu396Lys p.Glu840Lys > 10 bp	Damaging	EUR	$P = 6.1 \times 10^{-5}$ OR = 1.10 (95% CI = 0.98–1.23)
			Benign	ALL	$P = 1.1 \times 10^{-5}$ OR = 0.00 (95% CI = 0.00–0.04)

\*Unmeasurable; EUR: 379 European individuals from the 1000 Genome Project; ALL: all 1092 individuals from the 1000 Genome Project.

**Table 2** Nineteen SNVs that lost statistical power after the correction

Gene	SNV	Location/distance to splice	SIFT/PPH2	Population	Alelic P-value/OR (CI)
<i>PDE1C</i>	rs115957738	p.Asp122 = > 10 bp	*	EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE1C</i>	rs116449276	Intron > 10 bp	*	ALL	<i>P</i> =0.184 OR = 0.18 (95% CI = 0.02–1.46)
<i>PDE1C</i>	rs76290787	p.Ser175 = > 10 bp	*	EUR	<i>P</i> =0.001 OR = 1.06 (95% CI = 0.97–1.16)
<i>PDE1C</i>	rs78021925	Intron > 10 bp	*	ALL	<i>P</i> =0.008 OR = 0.05 (95% CI = 0.01–0.27)
<i>PDE2A</i>	rs392818	utr-3 > 10 bp	*	EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE2A</i>	rs11824084	utr-3 > 10 bp	*	ALL	<i>P</i> =0.452 OR = 1.72 (95% CI = 0.23–12.9)
<i>PDE2A</i>	rs4944557	p.Asp572 = 9 bp	*	EUR	<i>P</i> =0.001 OR = 1.06 (95% CI = 0.97–1.16)
<i>PDE4B</i>	rs34492439	p.Val388 = > 10 bp	*	ALL	<i>P</i> =0.381 OR = 0.63 (95% CI = 0.14–2.70)
<i>PDE4B</i>	rs114408173	Intron > 10 bp	*	EUR	<i>P</i> =0.003 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE4B</i>	rs7534999	c.604-3 C>T 3 bp	*	ALL	<i>P</i> =**
<i>PDE5A</i>	rs3733526	p.Ala93Val > 10 bp	Tolerated Benign	EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE5A</i>	rs114886951	p.Ile778Thr 2 bp	Tolerated Damaging	ALL	<i>P</i> =0.172 OR = 0.17 (95% CI = 0.02–1.35)
<i>PDE6A</i>	rs113309832	Intron > 10 bp	*	EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE6B</i>	rs61734864	p.Val835 = 2 bp	*	ALL	<i>P</i> =0.295 OR = 0.32 (95% CI = 0.04–2.52)
<i>PDE6B</i>	rs73058408	c.1722+5 G>A 5 bp	*	EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE6C</i>	rs193276411	Intron > 10 bp	*	ALL	<i>P</i> =0.014 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE6C</i>	rs76999928	p.Asp157Glu 10 bp	Tolerated Damaging	EUR	<i>P</i> =0.148 OR = 0.14 (95% CI = 0.01–1.14)
<i>PDE8A</i>	rs148254104	p.Leu350 = > 10 bp	*	ALL	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
<i>PDE8B</i>	rs182775622	p.Ala375 = > 10 bp	*	EUR	<i>P</i> =0.011 OR = 0.00 (95% CI = 0.00–0.08)
				ALL	<i>P</i> =**
				EUR	<i>P</i> =0.040 OR = 1.03 (95% CI = 0.96–1.09)
				ALL	<i>P</i> =0.028 OR = 0.01 (95% CI = 0.00–0.23)

\*Unmeasurable; \*\*absent data. EUR: 379 European individuals from the 1000 Genome Project; ALL: all 1092 individuals from the 1000 Genome Project.

## Discussion

In this work, we describe the presence of four novel variants in the PDE genes that might be associated with PCa development and/or progression. We also report three known SNVs to be significantly more frequent in PCa patients; an additional total of 19 (*P*-value < 0.05 when analyzed individually) were not significant after the correction. In addition, we report for the first time 35% higher occurrence of nsSNVs (*P*-value = 0.07996) in patients as compared to controls. As an index of function of these variants, the pCREB:CREB ratio was significantly higher in patients with high number of non-significant PDE variants (*P* < 0.001).

Our group has previously reported on the possible involvement of *PDE11A* in PCa pathogenesis (Faucz *et al.* 2011). Recent findings from other groups support these observations. To date, three PDEs, PDE4 (Kashiwagi *et al.* 2012, Henderson *et al.* 2014, Sarwar *et al.* 2014), PDE5, and PDE11 (Chavez *et al.* 2013, Hamilton *et al.* 2013) have been associated with PCa.

According to Sarwar *et al.* (2014), PDE4 inhibitors (PDE4i) play important roles in the AR/PSA expression pathway (Sarwar *et al.* 2014). It was discovered that the use of rolipram, a selective PDE4 inhibitor, enhances the production of cAMP/PKA and increases AR and PSA expression significantly (Sarwar *et al.* 2014). Prostatic

**Table 3** Four novel mutations in prostate cancer

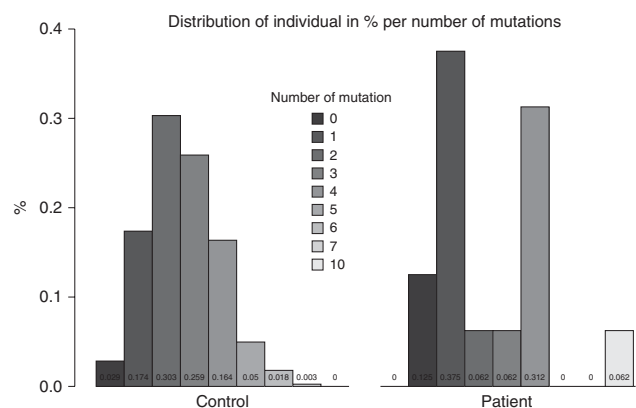
Gene	Chr	Position (hg18)	Geno type	Frequency	Distance to splice	Location	SIFT	PPH2	Mutation Taster	Sample origin
<i>PDE10A</i>	chr6	165782464	0/1	3 patients	9	c.347-9 A>C	*	*	Disease causing	Somatic
<i>PDE4B</i>	chr1	66231291	0/1	1 patient	123	p.Asn38Lys	Tolerated	Benign	Polymorphism	Somatic
<i>PDE6C</i>	chr10	95408566	0/1	1 patient	80	Intronic	*	*	Polymorphism	Germline
<i>PDE7B</i>	chr6	136536633	0/1	1 patient	6	p.Met239Ile	Tolerated	Benign	Disease causing	Germline

\*Unmeasurable; Chr: chromosome.

tissue is indeed highly sensitive to cAMP (Faucz *et al.* 2011), and both PKA activity and cAMP levels have been associated with PCa (Dimitriadis *et al.* 2008, Chung *et al.* 2009, Desiniotis *et al.* 2010, Sarwar *et al.* 2014). Our finding in this study that a novel, somatic nonsynonymous *PDE4B* variant (p.Asn38Lys) is present in PCa patients, may explain the down-regulation of *PDE4B* in PCa that has been described by others (Kashiwagi *et al.* 2012).

*PDE5A* does not seem to be influenced by the AR (Yang *et al.* 2009). A handful of recent studies have provided new insight on the possible therapeutic effects of *PDE5i* in PCA. Chavez *et al.* (2013) showed that men with erectile dysfunction treated with *PDE5i* tended to have reduced chance of developing PCa when compared to those who did not receive treatment. Two additional groups have reported that the compound found in green tea (epigallocatechin gallate) together with *PDE5i*, might stimulate apoptosis in PCa cells (Kumazoe *et al.* 2013, Yang & Wang 2013). Even though the rs114886951 lost its statistical power after Bonferroni correction, *in silico* analysis of this SNV seems to show a promising therapeutic target. This variation is located in the 100% splice site domain, *in silico* analysis shows complete loss of splice. Furthermore, this amino acid is highly conserved among different species (Mutationtaster; Schwarz *et al.* 2014). In our work, this variant was present in two individuals within the patient group (4.2%), whereas it was found in only one individual within the 1092 used in the project genome (0.0009%,  $P=0.034$ ; OR=16.1, 95%; CI=1.44–179). The higher reported frequency of this SNV was by the CSAgilent project (0.015%) whereas the ExAC project showed a similar frequency to the 1000 genome (0.001228%). It is noteworthy that this nsSNV (p.Ile778Thr) is located at the end of the catalytic domain of *PDE5*, within an  $\alpha$ -helix secondary structure of the protein core and it may destabilize the protein structure, in agreement to the *in silico* prediction for a damaging effect by SIFT (Ryan *et al.* 2009).

Even though *PDE11A* is capable of binding both cGMP and cAMP, it is important to highlight that *PDE11A* and *PDE5A* structures are quite similar (Fawcett *et al.* 2000). Both *PDEs* are responsible for the inhibition of 86% of the free cGMP in PCa cell lines (Hamilton *et al.* 2013), and together they are responsible for reducing the intracellular levels of PKG, a protein kinase involved in cell proliferation, the MAP kinase pathway, migration and neo-vessel growth in some types of endothelial cells (Hood & Granger 1998, Pyriochou, *et al.* 2007). Tadalafil, a *PDE5i*, binds relatively strongly to *PDE11* and reduces its activity (Pomara & Morelli 2005). While Chavez *et al.* (2013) showed that *PDE5i* inhibitors may have a protective effect from PCa, studies of *PDE11A* inactivation suggest opposite effects in various tumor settings (Horvath *et al.* 2006a,b,c, 2009, Boikos *et al.* 2008, Libe *et al.* 2008, 2011, Faucz *et al.* 2011, Vezzosi *et al.* 2012). According to Faucz *et al.* (2011), patients with PCa tended to have a higher frequency of *PDE11A*-inactivating variants when compared to controls ( $P$ -value <0.001; OR=3.81, 95%; CI=1.86–7.81). The present work confirms these findings and also adds two

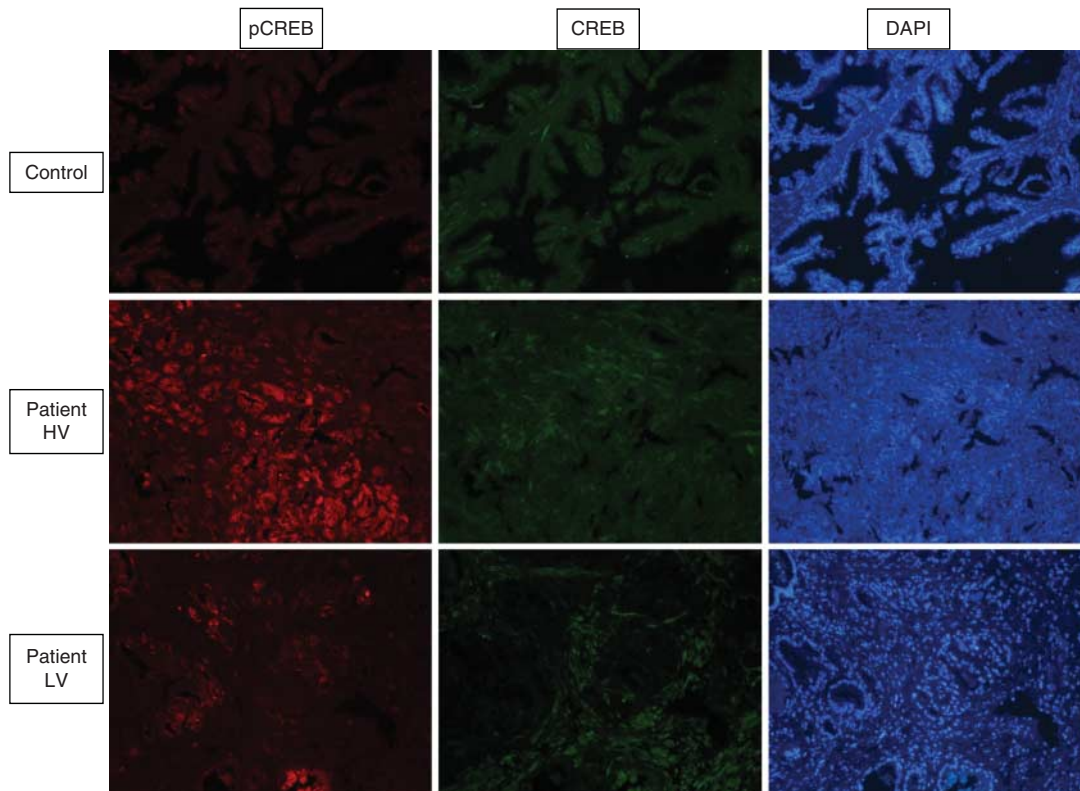


**Figure 1**  
*PDE11A* sequence variants and patients: side-by-side barplot expressing the percent of individuals distributed by the number of nonsynonymous variants encountered in each sample.

**Table 4** Presence of mutations, nsSNV or significant associated in each of the 16 tumor samples run through SOLiD

	Novel	PDE1C	PDE2A	PDE4A	PDE4B	PDE4C	PDE5A	PDE6A	PDE6B	PDE6C	PDE7A	PDE8A	PDE8B	PDE11A
DT01		<sup>a</sup> rs115957738	<sup>a</sup> rs577536 <sup>a</sup> rs11824084 <sup>a</sup> rs4944557			<sup>c</sup> rs2229228	<sup>b</sup> rs114886951		<sup>c</sup> rs115775983	<sup>c</sup> rs701865				<sup>b</sup> rs79903863 <sup>a</sup> rs75461311
DT02			<sup>a</sup> rs392818 <sup>a</sup> rs11824084			<sup>c</sup> rs2229228			<sup>a</sup> rs193276411 <sup>c</sup> rs701865 <sup>c</sup> rs701865					<sup>a</sup> rs75461311 <sup>a</sup> rs75461311
DT03	PDE6C	<sup>a</sup> rs76290787 <sup>a</sup> rs78021925		<sup>c</sup> rs1051738	<sup>a</sup> rs114408173	<sup>c</sup> rs2229228								<sup>a</sup> rs75461311
DT04														
DT07	PDE10A		<sup>a</sup> rs4944557			<sup>c</sup> rs2229228				<sup>c</sup> rs701865			<sup>c</sup> rs115599001	<sup>b</sup> rs79903863
	PDE7B													
DT08						<sup>c</sup> rs2229228				<sup>c</sup> rs701865			<sup>c</sup> rs115599001	<sup>b</sup> rs79903863
DT10						<sup>c</sup> rs2229228				<sup>c</sup> rs701865	<sup>c</sup> rs11557049		<sup>c</sup> rs115599001	<sup>c</sup> rs75672043 <sup>a</sup> rs75461311 <sup>c</sup> rs77597060 <sup>c</sup> rs17400325
DT12	PDE10A				<sup>c</sup> rs75398902	<sup>c</sup> rs2229228								
DT18							<sup>b</sup> rs3733526			<sup>c</sup> rs701865 <sup>b</sup> rs7699928 <sup>c</sup> rs701865				<sup>c</sup> rs75127279
DT19	PDE4B													
DT21		<sup>c</sup> rs61736729				<sup>c</sup> rs2229228	<sup>b</sup> rs3733526 <sup>c</sup> rs17051276 <sup>b</sup> rs3733526							
DT22				<sup>c</sup> rs1051738						<sup>c</sup> rs701865	<sup>c</sup> rs11557049			
DT32										<sup>c</sup> rs701865				
DT35			<sup>a</sup> rs577536			<sup>c</sup> rs2229228				<sup>c</sup> rs701865				
DT38		<sup>a</sup> rs116449276			<sup>a</sup> rs34492439 <sup>a</sup> rs7534999	<sup>c</sup> rs2229228		<sup>a</sup> rs113309832	<sup>a</sup> rs73058408 <sup>a</sup> rs61734864	<sup>c</sup> rs701865		<sup>a</sup> rs148254104		
DT45	PDE10A	<sup>a</sup> rs116449276 <sup>c</sup> rs61745829		<sup>c</sup> rs1051738		<sup>c</sup> rs2229228	<sup>b</sup> rs3733526 <sup>c</sup> rs17051276		<sup>c</sup> rs115775983	<sup>c</sup> rs4552236 <sup>c</sup> rs701865			<sup>a</sup> rs182775622	<sup>c</sup> rs75672043 <sup>c</sup> rs17400325

<sup>a</sup>Significant synonymous or intronic SNV.<sup>b</sup>Significant and nonsynonymous SNV.<sup>c</sup>Nonsynonymous SNV.

**Figure 2**

Immunohistochemistry (IHC) for the CREB (green) and pCREB (red) proteins in prostate tissue (10× magnification). Primary antibodies of rabbit anti-CREB and mouse anti-pCREB (dilution 1:100) and secondary antibodies goat anti-rabbit 488 and goat anti-mouse 555 were used to achieve this

image. Higher levels of pCREB (red) are observed in PCa tissue when compared to a control; there does not appear to be a difference in unphosphorylated CREB (green). Patient (HV), high number of PDE variants; patient (LV), low number of PDE variants.

significantly associated variants that are overrepresented in the PCa patients: rs79903863 ( $P$ -value  $\approx 6.1 \times 10^{-5}$ ; OR=1.10; 95% CI=0.98–1.23) and rs75461311 ( $P$ -value  $\approx 1.4 \times 10^{-4}$ ; OR=26.9; 95% CI=6.40–113).

Together with PDE11, PDE2 enzymes are both capable of degrading cAMP and cGMP at the same rate. Although there is no bibliographical references linking these PDEs to PCa, both are expressed in the prostate and have already been studied for their possible association with cancer (Wheeler *et al.* 2005, Traka *et al.* 2008, Lukk *et al.* 2010, Abusnina *et al.* 2011, Durand *et al.* 2011, Zhang *et al.* 2012, Morita *et al.* 2013). With similar results to our findings in PDE11A, our statistical analysis found one significant SNV

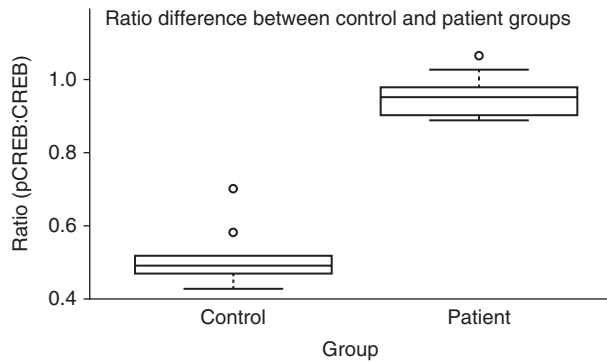
in PDE2A rs577536 ( $P$ -value  $\approx 6.1 \times 10^{-5}$ ; OR=1.10; 95% CI=0.98–1.23).

*PDE10A* was the second gene in which we found an interesting, frequent ( $\approx 19\%$ ) and novel somatic mutation. Like most of PDEs previously outlined, there is no report of *PDE10A* genetic variants in any type of cancer. A recent review described the use of PDE10 inhibitors for schizophrenia and neuropsychiatric disorders (Azevedo *et al.* 2014). Although *PDE10A* is mainly expressed in the brain, it is also present in the testis and prostate (Fujishige *et al.* 1999, Wheeler *et al.* 2005). Furthermore, *in silico* analysis of this novel variant shows a possible alteration in the splice site of the fifth exon. This exon is responsible for encoding

**Table 5** IHC statistic result for pCREB:CREB ratio

	P-value	CI 95%	Mean patient	s.e. patients	Mean control	s.e. control
Ratio	$2.97 \times 10^{-8}$	−0.54 to −0.35	0.97	0.03	0.52	0.03
pCREB	$1.88 \times 10^{-5}$	−23.51 to −11.51	31.45	1.62	13.95	2.29
CREB	0.4418	−17.37 to 8.28	32.44	1.17	27.89	5.52



**Figure 3**

Ratio difference between control and patient groups: a boxplot of the results for the analysis of fluorescence with a Leica TCS SP5 laser scanning confocal microscope. The results showed increased pCREB and pCREB:CREB ratio. Patients had a mean ratio of 0.97 with a standard error of 0.03, whereas controls had a mean of 0.52 with a standard error of 0.03. The boxplot also shows the presence of two outliers in the control group and one in the patients group.

the first 17% of the PDE10 first GAF domain. GAF's function in PDE10 remains controversial. It has been suggested that these domains bind to, and are activated by cAMP, which paradoxically results in potent competitive inhibition of cGMP turnover by cAMP (Jager *et al.* 2012). Even though this splice site variation is not located between the two 100% conserved cleavage sites, other variations with a similar distance have already been reported to cause mRNA changes and complete exon skipping (Fernandez Alanis *et al.* 2012). Furthermore, this site could be a possible adenine branch site (Pagani & Baralle 2004).

PDE7B has been explored for treatment of neurological diseases. Unlike most PDEs, PDE7B expression is minimal or absent in the prostate (Gardner *et al.* 2000, Hetman *et al.* 2000). However, it is activated through the cAMP/PKA/CREB pathway. The novel variant p.Met239Ile is located 6 bp from the most nearby exon boundary and its effects in prostate cells are worthy of *in vitro* studies.

Interestingly, we found in our patients three previously described variants that show association with testicular or adrenal tissues. The PDE11A variants rs17400325 and rs75127279 were found in three patients with bilateral testicular germ cell tumors (Horvath *et al.* 2006b). The PDE8B variant rs115599001 was found in one patient with adrenal cancer (Rothenbuhler *et al.* 2012) and in three of our patients, with a tendency of significance when compared with the ALL population ( $P=0.021$ ; OR=0.09, 95%; CI=0.02–0.44).

Finally, analysis of the pCREB:CREB ratio has been shown to be a good indicator of the amount of cAMP

present in any tissue. Kaname *et al.* (2014) showed that an increase in PDE4A and PDE4B levels might cause low cAMP accumulation and a decrease in the pCREB:CREB ratio. A different study showed that PDE4i is capable of reversing this effect through up-regulation of cAMP and increasing pCREB:CREB ratio (Guo *et al.* 2014). Our IHC experiment showed an increase of the ratio (1.86× higher when compared to control) similar to the results obtained by the group that used PDE4i. Kumar *et al.* (2007) had previously reported similar results in the prostate and suggested its potential role as a prognostic marker.

How the inhibitors selectively bind the conserved PDE catalytic domains is unknown for most PDEs (Huai *et al.* 2003). A single report suggested that the rolipram inhibitor selectivity on PDE4 is determined by the chemical nature of amino acids and also describes a relationship between the insensitivity of this drug with PDE7A genetic variants (Huai *et al.* 2003). Maybe this same principle can be expanded to other inhibitors, to produce different outcomes depending in each tissue and disease. In addition, higher intracellular levels of cGMP have been reported to 'crossover' and activate this kinase (Cornwell *et al.* 1994, Hood & Granger 1998). Over-expression of PKA has already been associated with PCa (Desiniotis *et al.* 2010, Merkle & Hoffmann 2011) and other types of cancers (Horvath *et al.* 2006a,c, 2008, 2010, Stratakis *et al.* 2010, Libe *et al.* 2011, Almeida *et al.* 2012).

We conclude that various PDE genetic variants may be associated with predisposition and/or progression to PCa. Understanding the molecular signaling basis of this association could be key to the development of novel therapeutic approaches for this common form of cancer.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0134>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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