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RESEARCH ARTICLE

Promoter polymorphisms of the *PCA3* gene are not associated with its overexpression in prostate cancer patients

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Abstract. In male, the prostate cancer (PCa) is one of the most frequent neoplasias and the second cause of cancer deaths worldwide. In 2015, more than 6000 men died in Mexico due to this disease. In this regard, prostate cancer associated gene 3 (*PCA3*) has become an interesting target in PCa as is found highly overexpressed. Moreover, TAAA tandem repeats have been suggested to be associated with the regulation of *PCA3* expression and, in turn, to be related with the development of the disease. The aim of the study was to understand the genetic basis of the disease in search for a better diagnosis. Expression levels of *PCA3* gene were analysed in tissue of 13 patients diagnosed with PCa and six patients diagnosed with a benign prostatic disease (BPD). The absolute expression of *PCA3* was quantified by real-time PCR. Genotype for TAAA tandem repeats was measured using automatic sequencing and the results were analysed to determine whether an association existed between them. We identified three alleles: 4, 5, 6 and four genotypes: 4/5, 5/5, 5/6, 6/6. Our analysis identified a mutation in the nucleotide 76764237 of the *PCA3* gene that generates an extra TAAA tandem repeat. The nucleotide mutation is present in 61.53% of PCa and 66.66% of BPD patients. Our study revealed the presence of a mutation in the *PCA3* gene that generates an extra TAAA tandem. We observed no association between the absolute expression of *PCA3* messenger and the number of TAAA repetitions.

Keywords. expression; genes; polymorphism; TAAA repeats; *PCA3*; prostate cancer.

Introduction

Worldwide, prostate cancer (PCa) occupies the second place in male cancer-related deaths (Torre *et al.* 2015). Particularly in Mexico, in the last decade, PCa has positioned as the main cause of cancer death in men and its incidence is believed to be increasing (INEGI 2016). Recently, a biomarker for PCa, prostate cancer-associated gene 3 (*PCA3*) was identified. *PCA3* offers high sensitivity and specificity unlike other biomarkers for PCa such as PSA protein. Further, *PCA3* expression is not influenced by the enlargement or any other benign diseases of the prostate (Bussemakers *et al.* 1999).

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PCA3 gene is composed of four exons and its approximated size is 25 Kb. It is located on chromosome 9q21.2 and its expression product is a noncoding mRNA (Bussemakers *et al*. 1999; Zhou *et al*. 2011, 2014). This gene does not possess conventional elements for transcription regulation. However, several studies have identified sub-regions in the promoter region, specifically from −433 to +62, containing a high number of *cis*-acting elements that are necessary for its expression. An example of these sub-regions is FP2, where short-tandem repeats (STRs) containing mostly adenines and thymines were identified (Verhaegh *et al*. 2000; Zhou *et al*. 2011, 2014).

The STRs corresponding to TAAA sequence show significant differences in the number of repetitions between patients with and without PCa. According to the NCBI database, it has been established that the wild-type allele is $(TAAA)_5$ (number five corresponds to the number of repetitions). In the *PCA3* gene, it is believed that each TAAA tandem repeat is a site for transcription initiation, thus proposing that these STRs are part of its promoter region and therefore regulate the transcription rate (Zhou *et al*. 2011, 2014).

Under this approach, the identified TAAA tandem repeats may be associated with the regulation of *PCA3* expression and, in turn, to be related to the development of the disease. The aim of the study was to determine the relationship between *PCA3* expression and the genotypes of TAAA tandem as this may serve as a tool for diagnosis of PCa.

Material and methods

Study participants

This study included a total of 19 patients, 13 diagnosed with PCa (aged 67.5 ± 7.9 years) and six diagnosed with benign prostatic disease cases (BPD) (aged 66.6 ± 5.08 years). Volunteers were recruited from the 'Álvarez & Arrazola Radiólogos' clinic and the 'Instituto Mexicano del Seguro Social', in Sinaloa, México, between August 2016 and October 2017. A written consent was obtained from each participant and the clinical data was obtained by direct questionnaires. This study protocol was reviewed and approved by the ethics committee of above-mentioned health institutions.

Histopathological analysis

Tissue samples were obtained by a trained professional via biopsy. Samples were analysed by an experienced pathologist, who provided a complete report for each patient about tumour differentiation at a cellular level. Results included the indication of tumour aggressiveness according to Gleason score.

Real-time PCR absolute expression

For molecular analysis, tissue samples were preserved in 750 μL of trizol, from where genomic RNA was extracted and purified with the MiRNeasy Mini, Qiagen kit according to the protocol provided by the manufacturer. For *PCA3* expression analysis, cDNA was synthesized from RNA samples with the ImProm-II Reverse Transcription System kit (Promega Corporation, Madison, USA). Quantification was performed by real-time PCR (StepOneplus system of Applied Biosystems) using Taqman probes with FAM label (Applied Biosystems, Foster City, USA) to quantify the absolute gene expression in the tissue. Finally, the number of copies of *PCA3* present in the cDNA was calculated.

Fragment analysis

An injection mixture of 1 μL of PCR product, 0.25 μL of standard size GS500 ROX and 12 μL of HiDi formamide was prepared. Following denaturation with cycles of 93°C for 2 min and ice for 2 min, the mixture was injected into an ABI PRISM 310 Genetic Analyzer (from Applied Biosystems) and run under the following conditions: dye primer FAM4, capillary 30 cm, injection voltage at 15 kV, injection time 5 s, run time 25 min, filter set F and POP4 polymer. The size of the wild-type TAAA repeat (349 bp) was taken as a reference to discriminate the number of repetitions of all samples.

Sequencing analysis

For automated sequencing, genomic DNA was extracted from peripheral blood of PCa and BPD patients using Gustincich *et al*. (1991) method. *PCA3* promoter was amplified by PCR under standard condition using a T100 thermal cycler (from Biorad) with primers mentioned elsewhere (Zhou *et al*. 2014). PCR was performed in 50 mL of reaction mix containing 5 μL DNA extract, 10 μL *Taq* polymerase buffer 5x, 6 μL $MgCl_2$ (25 mM), 1 μL dNTP (10 mM), 0.5 μL of each forward and reverse primer (10 mM), 0.2 μL *Taq*-polymerase and 26.8 μL distilled water with conditions as follow: 94°C for 5 min, 35 cycles of 94°C for 1 min, 35 cycles of 55°C for 1 min, 35 cycles of 72°C for 1.5 min and finally, 72°C for 5 min. All PCR products were electrophoresed on a 1.5% agarose gel. Amplified fragments were sent to Macrogen Corporation in Seoul, Korea for sequencing confirmation.

Statistical analysis

Statistical analysis was performed with the statistical package for social sciences for Windows (v. 20.0; SPSS). The

normality of the data was verified by means of the Shapiro–Wilks statistical test. The Student's *t*-statistic was used to assess the distribution of the samples. Analysis of variance (ANOVA) and the Tukey significance test was carried out to determine the significance of subgroups. Significance was defined as *P* values of 2-tailed tests lower than 0.05.

Results

Clinical and histopathological results

Body mass index (BMI) is a character strongly related to chronic diseases, however, our results showed no significant difference ($P = 0.473$) between the PCa and BDP group (average of 25.74 ± 1.00 and 24.78 ± 0.79, respectively). As per serum PSA levels, PCa patients had an average of 102.7 ± 214.9 ng/mL (ranging from 4.29 to 726 ng/mL) and BDP patients an average of 17.15 ± 1.5 ng/mL (ranging from 16 to 18.3 ng/mL), which in turn did not represent a difference statistically significant ($P = 0.513$). Additionally, we analysed the relationship of PSA levels with respect to BMI where no relationship was observed ($P = 0.987$, Rho = − 0.006).

With respect to histopathological analysis, according to the Gleason score, we identified four different scores in PCa patients, 6 (3 + 3), 9 (5 + 4), 7 (3 + 4) and 7 (4 + 3) with frequencies of 10%, 20%, 30% and 40%, respectively. ANOVA between the obtained serum PSA levels and Gleason scores yielded significant differences when compared with the groups that scored 7 (3 + 4) ($P = 0.039$) and 9 (5 + 4) ($P = 0.026$).

Expression analysis

With respect to absolute *PCA3* expression, the number of copies was calculated with an average for PCa patients of $4.1\text{x}10^7 \pm 2.8\text{x}10^7$ copies/tissue μg, and an average of $2.2 \times 10^6 \pm 3.5 \times 10^6$ copies/tissue μg for BPD, a significant difference was observed between the groups in our study population ($P = 0.027$). Given their importance in the detection of PCa, the correlation between the number of *PCA3* messenger copies and the serum PSA levels was analysed. After Spearman's statistical correlation analysis, we found an existing relationship between these two biomarkers ($P = 0.027$). In addition, we observed that this correlation is moderately and directly proportional (Rho = 0.578).

Fragment and sequencing analysis

In this study, we obtained the sizes of TAAA repeats by fragment analysis. Two sizes were determined in our population, 349 bp and 345 bp, the latter corresponding to a deletion of a TAAA repeat (figure 1). The deletion was observed in 30.76% of PCa and 16.66% of BPD patients.

According to sequence analysis, we identified alleles 4, 5, 6, (figure 2) and genotypes 4/5, 5/5, 5/6, 6/6, which were then classified as groups I, II, III and IV, respectively. In group I, the results revealed the deletion of a TAAA tandem repeat. Likewise, in groups III and IV, our analysis identified a mutation at the position chr9:76764237 of the *PCA3* gene which generates the change [C/T] that produces an extra TAAA tandem repeat. This mutation corresponds to the rs10869813 polymorphism. The frequency in our population of T allele was 0.34. Further, group I deletion was present in 30.76% of PCa and 16.66% of BPD patients and groups III and IV nucleotide mutation was present in 61.53% of PCa and 66.66% of BPD patients. On the other hand, the wild-type TAAA sequence was observed in 16.66% of BPD patients and absent in patients with PCa. No statistical difference was found between serum PSA levels and number of *PCA3* messenger copies (table 1) in the pooled genotypes. Lastly, there was no association between genotypes and Gleason score in PCa patients (table 2).

Discussions

PSA is currently used as a tumour marker for early diagnosis and in estimating tumour burden and clinical stage of PCa. In the present study, a difference was observed in serum PSA levels between groups of Gleason 7 (3 + 4) and Gleason 9 (5 + 4). In 2006, Elabbady and Kherd (2006) conducted a study where they associated PSA levels with the Gleason score in PCa patients, they concluded that the higher the serum PSA level, the more aggressiveness they denoted in the Gleason score. In our population, the highest serum PSA levels were located within the Gleason 9 (5 + 4) (89.61 ng/mL, average value) and Gleason 7 (4 + 3) (39.82 ng/mL, average value) groups. Therefore, we reinforced the positive correlation between both clinicopathological variables and showed its implication as a diagnostic tool (Berger *et al.* 2005; Elabbady and Kherd 2006).

We found a higher expression of *PCA3* in PCa compared to BPD patients, supporting previous studies (Bussemakers *et al.* 1999; Marks and Bostwick 2008; Tao *et al.* 2010) where a higher number of copies of *PCA3* messenger were found in patients with PCa. Moreover, we found a moderate and directly proportional correlation between the number of copies and serum PSA levels. These results provide more evidence about the importance of serum PSA levels and *PCA3* expression in the detection and prognosis of PCa (Marks and Bostwick 2008; Neves *et al.* 2013). However, in clinical evaluations, *PCA3* expression level remains unaltered during noncancerous pathologies whereas PSA is commonly found elevated (Marks *et al.* 2007). Therefore, *PCA3* gene expression may be useful to discriminate between PCa and BPD.

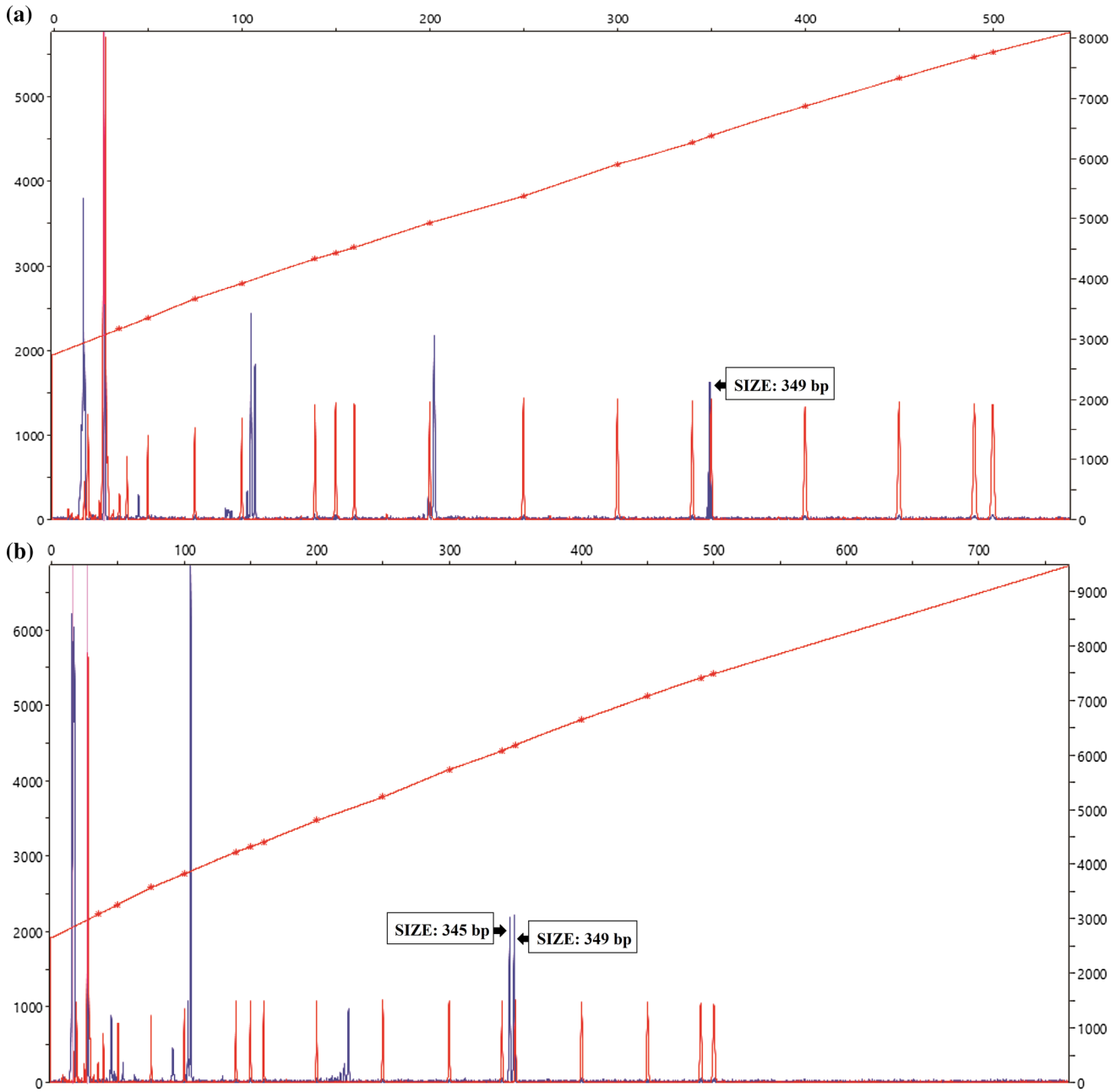

Figure 1. Fragment analysis of TAAA repeats. On the *x*-axis, the corresponding fragment size is observed on the *y*-axis the height of each peak is shown. (a) The size of 345 bp corresponds to allele 4. (b) The size of 349 bp corresponds to allele 5 (wild-type).

Studying the genetic alterations of the *PCA3* gene may be useful to establish the pathogenesis of PCa. In the present study, we screened mutations in the *PCA3* gene, specifically for the number of TAAA tandem repeats. Sequencing analysis identified the presence of three alleles (4, 5 and 6) and four genotypes (4/5, 5/5, 5/6 and 6/6) in our patients. Zhou *et al.* (2011) identified five alleles (4, 5, 6, 7 and 8) and eight genotypes (4/5, 4/6, 5/5, 5/6, 5/7, 5/8, 6/6 and 6/7) in Chinese population. However, some genotypes identified in our study (5/6 and 6/6) are caused due to a mutation in the *PCA3* gene that produces a change of the nucleotide [C/T]. In contrast, in a study conducted in Asian population the resulting genotypes were due to insertions of TAAA tandem repetitions. Interestingly, Zhou *et al.* (2014) showed significant differences between genotypes of patients with and without PCa, suggesting that PCa incidence is closely related to the numbers of TAAA tandem repeats in the promoter region of *PCA3* gene and therefore associated with an increased risk for prostate cancer in Chinese population. We decided to investigate the role of TAAA tandem in the genetic expression of *PCA3*. However, in the genotypes that we identified, the number of TAAA tandem repeats was not

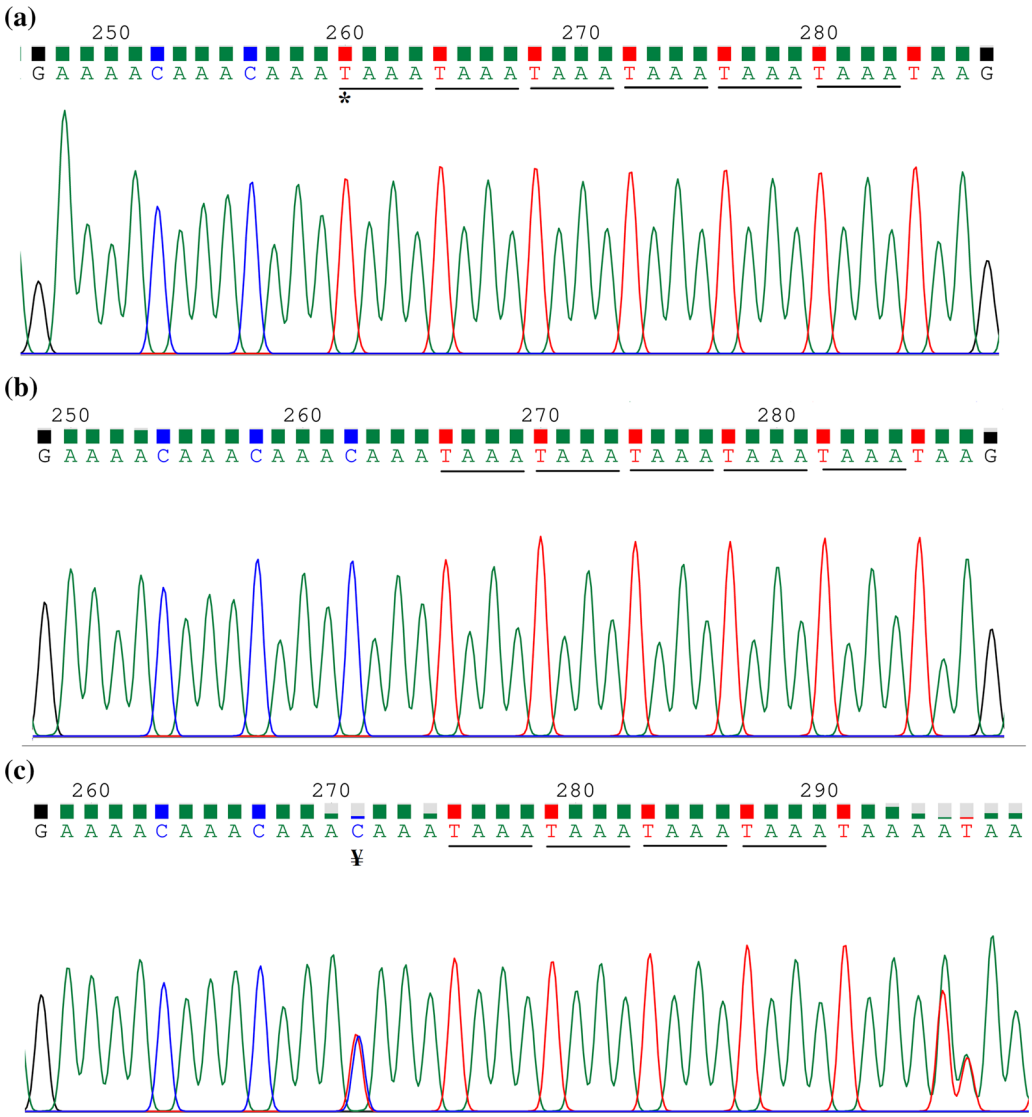

Figure 2. Representative sequencing of STR polymorphism in the promoter region of the *PCA3* gene. (a) Allele 6; (b) allele 5 (wild-type); (c) allele 4. *Position of the mutated nucleotide [C/T] (homozygous). ¥Position of the mutated nucleotide [C/T] (heterozygous).

Table 1. Association between genotype groups and clinicopathological characteristics.

	PCA3 messenger			Serum PSA levels mg/mL (average)		
Group	PCa	BPD	*P* value	PCa	BPD	*P* value
I	1.6×10^6	4.8×10^4	0.593	10.1	18.3	0.432
II	2.8×10^8	1.1×10^5		88	–	
III	3.1×10^7	2.6×10^6		144.61	16	
IV	–	5.3×10^6		–	–	

PCa, patients diagnosed with prostate cancer; BPD, patients diagnosed with a benign prostatic disease.

associated with changes in the *PCA3* gene expression. Although in previous studies, the induction of mutations in PCa cell lines resulted in a decreased transcription rate of *PCA3*, identifying it as a possible promoter region (Verhaegh *et al*. 2000). Nonetheless, these studies did not analyse the gene expression. It has been observed that overexpression of

Table 2. Association between genotype groups and Gleason score.

	Gleason score				
Group	6 (3 + 3)	7 (3 + 4)	7 (4 + 3)	9 (5 + 4)	*P* value
I	1	2	1	–	0.633
II	–	–	1	–	
III	1	2	1	1	
IV	–	–	–	–	

PCA3 gene is associated with the development of PCa. However the mutations found in the gene were not associated with any change in the expression. This means that although there are mutations in the *PCA3* gene, they do not alter the prognostic value as a biomarker for the disease.

In addition, we identified the presence of rs10869813 polymorphism which was previously identified by massive sequencing during the 1000 Genomes Project phase 3, however, its effects remained unknown. Importantly, we discovered that this polymorphism causes an extra TAAA repetition and that the frequency of T allele in our population was 0.34. This is in accordance with the south Asia and Europe population, where the presence of the mutated T allele has been reported with frequencies of 0.35 and 0.32, respectively. Moreover, Mexico is a country where miscegenation prevails, mainly between indigenous and European lines, so that in this population there is great European contribution. On the other hand, Mexicans are an admixture of different races such as Asian, which may explain these similarities in allelic frequencies (Rangel *et al*. 2008; Rubí *et al*. 2009; Silva *et al*. 2009).

It has been observed that genotypes with high numbers (5/6, 5/7, 5/8, 6/6, 6/7) also present an increased number of TAAA repetitions and ultimately, this is related to an increased risk of developing PCa (Zhou *et al*. 2014). With respect to our study, we also found two genotypes that follow this same behaviour (5/6 and 6/6). It is important to highlight that this is the first study conducted in Mexican population involving TAAA repeats. Further studies are needed to determine the most frequent genotypes in this population as Mexicans present a high heterogeneity.

In conclusion, our study revealed that in Mexican population the distribution of genotypes TAAA differs between individuals. Ultimately, no association was found between the number of TAAA repeats and the regulation of *PCA3* expression.

The genotypes with a higher number of repetitions were found in patients with PCa. However, we identified in Mexican men the presence of rs10869813 polymorphism in the *PCA3* gene promoter that generates an extra TAAA repeat. Finally, it was observed that the *PCA3* overexpression is not caused by the mutations, this is caused by the pathology.

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