Genetics and Molecular Research

Expression of PCA3 and PSA genes as a biomarker for differential diagnosis of nodular hyperplasia and prostate cancer

F. Fonseca Coelho[1,2], F. Loli Guimarães[2], W.L. Ribeiro Cabral[3], P.G. Oliveira Salles[3], E. Cueva Mateo[4], L. Mendes Nogueira e Nogueira[5], C.E. Corradi Fonseca[5] and K. Braga Gomes[1,2]

[1]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[3]Instituto Mario Penna, Belo Horizonte, MG, Brasil
[4]Instituto Hermes Pardini, Belo Horizonte, MG, Brasil
[5]Hospital das Clínicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Corresponding author: K. Braga Gomes
E-mail: karinabgb@gmail.com

Genet. Mol. Res. 14 (4): 13519-13531 (2015)
Received March 10, 2015
Accepted June 17, 2015
Published October 28, 2015
DOI http://dx.doi.org/10.4238/2015.October.28.13

ABSTRACT. We evaluated the expression of the *PCA3* gene in urine from patients with nodular hyperplasia/benign prostatic hyperplasia (PNH) or adenocarcinoma type prostate cancer (PCa).The study included 59 men: 22 with PCa, 26 with PNH, and 11 with no alterations (controls). Patients' urine was collected following prostatic massage and quantified by quantitative real-time PCR for prostate cancer antigen 3 gene (*PCA3*) and prostate-specific antigen gene (*PSA*) expression with the *ACTB* gene for normalization. *PCA3* gene expression was detected in 16 patients with PCa and 4 with PNH; in the control group, there was no expression of the gene. No significant difference was observed in the mean levels of *PCA3*

and *PSA* expression, the PCA3/PSA ratio, and the total PSA levels when the groups of patients with PCa and PNH were compared. The area under the receiver operating characteristic (ROC) curve was 0.625, 0.596, 0.559, and 0.503 for PCA3 and PSA expression, the PCA3/PSA ratio, and total PSA levels, respectively. The sensitivity and specificity of the PCA3 test were 73 and 85%, respectively. Considering the estimated cutoff values (0.2219 and 0.5007 for *PCA3* and PCA3/PSA, respectively), we observed a significant difference between the frequency of individuals with values above in the PCa group compared with the PNH group ($P < 0.001$). We conclude that the qualitative PCA3 test could be applied to initial screening for differentiation between individuals with PCa or PNH and those without prostate changes.

Key words: Prostate cancer; Nodular hyperplasia; *PCA3*; *PSA*; Gene expression

INTRODUCTION

Prostate cancer (PCa) is a complex disease and genetic factors contribute to its development, but the variants involved remain unknown. The most common type of PCa is adenocarcinoma, or glandular cancer, which particularly affects the peripheral zone (Abate-Shen and Shen, 2000; De Marzo et al., 2004; Nelson et al., 2004). The incidence of PCa increases with age, affecting mainly men over 50 years old, resulting in a major impact on health systems. An increased incidence has been observed in recent years owing to the exposure of the population to risk factors, the increase in life expectancy, the improvement and development of diagnostic methods, and the dissemination of screening by measurement of prostate-specific antigen (PSA) and digital rectal examination (MS/INCA, 2013).

Currently, screening for PCa is based on serum PSA measurement combined with clinical examination (digital rectal examination). When these preliminary tests suggest the presence of the disease, a transrectal prostate biopsy is indicated. The confirmatory diagnosis is performed by histological examination of the tissue obtained (NICE, 2008). The detection of serum PSA has emerged as a promising marker for PCa and is still widely used in screening for the disease. However, its levels increase in cancer and benign prostate changes, which make it an unspecific marker for PCa diagnosis. When alterations in PSA levels or nodules are observed, prostate biopsy, an invasive procedure, is recommended but often unnecessary (MS/INCA, 2013). The large number of patients with elevated PSA levels and negative biopsy is the major clinical problem in the current approach (ACS, 2012).

The serum PSA test coupled with needle biopsy is the standard clinical practice in prostate cancer diagnosis, but it is limited by the outcomes of excessive negative biopsies, overdiagnosis of clinically insignificant cancers, and a significant false-negative biopsy rate (Strope and Andriole, 2010; Sandblom et al., 2011).

The use of molecular markers is a new proposal for PCa diagnosis. It enables early diagnosis in addition to being more specific and less invasive. The new technologies allow detection of genetic abnormalities in prostate tissue, blood, urine, and body fluids, and include the study of genes that show higher expression levels in the presence of prostate tumor and low expression

levels in benign changes (Shappell, 2008). A large number of markers for PCa diagnosis have been studied, but one of them, prostate cancer antigen 3 gene (*PCA3*) has shown significant results in terms of diagnostic value (Hessels and Schalken, 2009).

The *PCA3* gene is located on the long arm of chromosome 9 (23 Kb) and comprises four exons (locus 9q21.2). Studies have shown that its mRNA contains a large number of termination codons and three open reading frames, suggesting that the gene does not code for proteins (Hessels and Schalken, 2009). In 1999, *PCA3* (also known as the *DD3* gene) was first identified in a study showing high expression levels in prostate tumor tissue compared with normal adjacent tissue from the same patient. The overexpression of the *PCA3* gene was observed in 95% of PCa samples, and there was no detection of gene expression in any other tumor or normal tissue from different organs. In non-tumor tissues, or in the presence of benign prostate changes, very low levels of *PCA3* gene expression were detected. The strong association between *PCA3* gene overexpression and malignant prostate changes has assigned an important role for this gene as a marker for PCa diagnosis (Bussemakers et al., 1999). The function of the *PCA3* gene is still unknown but studies have shown that non-coding RNA of this gene is involved in control of PCa cell survival, modulating cell signaling via androgen receptors, which could raise new possibilities for the use of this marker in PCa therapies (Ferreira et al., 2012).

This study evaluated the expression of the *PCA3* gene in PCa and prostatic nodular hyperplasia (PNH) patients compared with individuals with no prostate changes. The results could contribute to the standardization of a marker for the detection of the earliest forms of PCa in asymptomatic individuals, enabling immediate intervention and treatment of the disease. Individuals with benign changes may also benefit, avoiding repetitive biopsies if gene expression is not detected.

MATERIAL AND METHODS

Samples

The study included 59 men that had been referred to the Radiology Department of Hospital das Clínicas at Universidade Federal de Minas Gerais (HC-UFMG) for prostatic biopsy. The study included patients with adenocarcinoma-type PCa (N = 22), PNH (N = 26), and individuals with no pathologies (N = 11) as a control group. Diagnoses were established based on clinical and histopathological data supplied after the biopsy, and molecular testing comprising a double-blind study.

The samples were collected from February 2013 to February 2014. A sample of the urine (approximately 30 mL) taken immediately after prostatic massage was collected before the biopsy. A physician applied pressure to the prostate base towards the apex three times each for the left and the right lobes. This procedure releases the prostate epithelial cells into the urinary tract via the prostate duct.

The data collected included age, medical history, family history of PCa and other cancers, medications, smoking habits, urinary disorders, and serum PSA levels. For determination of cancer or PCa family history, relatives of the 1st to 3rd degree that had been diagnosed with the diseases were considered. The use of 5-alpha-reductase inhibitors, e.g., finasteride, which is well known to cause interference in laboratory tests, was investigated. We considered subjects to have a regular smoking habit if they had smoked any number of cigarettes for a period longer than six months in the past five years. The information not accessed in the medical records was obtained by patient interview.

RNA isolation and DNase treatment

The samples were immediately stored on ice for up to 30 min until centrifugation, which was performed at 1000 *g* for 10 min at 4°C. RNA later (Life Technologies®, Carlsbad, CA, USA) (1 mL) was added to the sediment obtained, which was stored at 2°-8°C for a maximum of 24 h until required for RNA extraction. Before extraction, the samples were washed with phosphate-buffered saline to remove the preservative and centrifuged at 5000 *g* for 10 min at 4°C. TRIzol (Life Technologies®) (1 mL) was added to the sediment, which was incubated at room temperature for 5 min. We then added 200 µL chloroform and continued centrifugation at 21,000 *g* for 15 min at 4°C. We then added 600 µL 100% isopropanol to the upper aqueous phase. The samples were kept at room temperature for 10 min; after another centrifugation at 21,000 *g* for 20 min, we washed them with 75% ethanol and centrifuged again at 21,000 *g* for 5 min. The dried pellets were eluted in 20 µL RNase-free water (Qiagen®, Hilden, Germany) and hydrated at 60°C for 10 min. After quantification of the samples in a NanoDrop spectrophotometer (Thermo®, Wilmington, MA, USA), we continued the treatment with DNase. We added the following mixture to 1000 ng RNA: 2 µL 10X DNase Buffer, 1.36 µL DNase I (Qiagen®), and RNase-free water (Qiagen®) to make the volume up to 20 µL. The digestion was carried out at 37°C for 30 min followed by inactivation at 65°C for 5 min.

Absolute quantification of the *PCA3* and *PSA* genes

We determined the expression of *PCA3* (assay ID Hs01371939_g1), *PSA* or *KLK3* (Human Kallikrein, assay ID Hs01371939_g1), and *ACTB* (β-actin, assay ID Hs01060665_g1) genes using an Absolute Quantification Real-Time PCR One-Step 7500 Fast® system (Applied BioSystems®, Foster City, CA, USA). We used the SuperScript III Platinum One-Step Quantitative reverse transcription polymerase chain reaction (RT-PCR) System (Invitrogen®) and TaqMan Assays (Applied BioSystems®).

A standard curve for the three target genes was validated. We used gBlocks Gene Fragments synthesized DNA fragments (Integrated DNA Technologies) for the *PSA* and *PCA3* curves (Table 1). We used the *ACTB* reference gene as endogenous control and a purified genomic DNA for the standard curve. Each standard curve point was separately analyzed in triplicate for each gene, while sample reactions were performed in multiplex.

Table 1. Sequences synthesized for construction of prostate cancer antigen 3 (*PCA3*) gene and prostate-specific antigen (*PSA*) gene standard curves.

Gene	Sequence
PSA (206 bp)	5'-ATGTGGGTCCCGGTTGTCTTCCTCACCCTGTCCGTGACGTGGATTGGTGCTGCACCCCTCATCCTGTCTCGGATTGT GGGAGGCTGGGAGTGCGAGAAGCATTCCCAACCCTGGCAGGTGCTTGTGGCCTCTCGTGGCAGGGCAGTCTGCGGC GGTGTTCTGGTGCACCCCCAGTGGGTCCTCACAGCTGCCCACTGCATCAGGAA-3'
PCA3 (500 bp)	5'-GTGAGAAATAAGAAAGGCTGCTGACTTTACCATCTGAGGCCACACATCTGCTGAAATGGAGATAATTAACATCACTAGA AACAGCAAGATGACAATATAATGTCTAAGTAGTGACATGTTTTTGCACATTTCCAGCCCCTTTAAATATCCACACACACAGG AAGCACAAAAGGAAGCACAGAGATCCCTGGGAGAAATGCCCGGCCGCCATCTTGGGTCATCGATGAGCCTCGCCCTGT GCCTGGTCCCGCTTGTGAGGGAAGGACATTAGAAAATGAATTGATGTGTTCCTTAAAGGATGGGCAGGAAAACAGATCCT GTTGTGGATATTTATTTGAACGGGATTACAGATTTGAAATGAAGTCACAAAGTGAGCATTACCAATGAGAGGAAAACAGAC GAGAAAATCTTGATGGCTTCACAAGACATGCAACAAACAAAATGGAATACTGTGATGACATGAGGCAGCCAAGCTGGGG AGGAGATAACCACGGGGCAGA-3'

Statistical analysis

Version 13.0 of the Statistical Package for Social Sciences (SPSS) was used for analyses.

We used the Shapiro-Wilk test to evaluate data normality. Nonparametric continuous variables are reported as median and interquartile range. Parametric continuous variables are reported as means ± standard deviation. The Kruskal-Wallis and Mann-Whitney tests were used for median comparison among three and two groups, respectively, for nonparametric variables. The means of three and two groups were compared by ANOVA followed by post hoc least significant difference or Student test, respectively, for parametric variables. The chi-square test or Fisher exact test was applied to compare the categorical variable frequencies. For significant differences by chi-square among three groups, residue testing was applied considering the least frequent variable to be less than -1.96 and the most frequent variable to be greater than 1.96. The receiver operating characteristic (ROC) curve was plotted to evaluate the sensitivity and specificity of the continuous variables in the diagnosis of prostate abnormalities. P values <0.05 were considered to be significant.

RESULTS

Clinical characterization

The characteristics of the groups with regards to demographics and risk factors, such as smoking habits, cancer family history (of any kind), PCa family history, and the use of 5-alpha reductase inhibitors, are shown in Table 2. No significant difference was observed in the frequency of these variables between the groups (P > 0.05). However, we observed a lower frequency of individuals with a family history of PCa in the control group compared with the other groups (P = 0.048).

Table 2. Clinical and demographic characterization of prostate cancer (PCa), prostatic nodular hyperplasia (PNH), and control groups.

Variable	PCa (N = 22)	PNH (N = 26)	Control (N = 11)	P
Age	64.87 ± 7.34	66.60 ± 7.84	66.00 ± 6.53	0.720
Smoking habit (yes)	9 (39.1%)	11 (44.0%)	4 (36.4%)	0.895
Cancer family history (yes)	11 (47.8%)	13 (52%)	4 (36.4%)	0.687
PCa family history (yes)	9 (39.1%)	6 (24.0%)	0 (0%)[a]	0.048*
Use of 5-alpha reductase inhibitors (yes)	2 (8.7%)	2 (8.0%)	0 (0%)	0.609

Age: means ± SD, analysis of variance. Other variables: absolute number (frequency), chi-square. [a]Less frequent by residue test. *Statistically significant.

Expression of the *PCA3* and *PSA* genes and total serum PSA

The quantification of *PCA3* and *PSA* gene expression normalized by the endogenous control, and the PCA3/PSA ratios were compared between the groups. Values of total serum PSA were obtained from medical records (Table 3). Sixteen patients had detectable expression values for *PCA3* and *PSA* genes in the PCa group. Only four and seven patients in the PNH group presented detectable *PCA3* and *PSA*, respectively. Thus, PCA3/PSA mean values were determined only for patients who had quantifiable levels of the *PCA3* gene. None of the controls presented a measurable value of *PCA3* expression and only one individual showed *PSA* gene expression. No significant difference was observed in the expression of *PCA3* and *PSA* genes or the PCA3/PSA ratio when we compared the PCa and PNH groups, although *PCA3* expression and PCA3/PSA ratio were higher in the PCa group (all P > 0.05). No significant difference was found relative to the total serum PSA level, although a tendency to higher values in the PCa group, followed by the PNH and control groups, was observed (P = 0.065).

Table 3. Normalized number of copies of prostate cancer antigen 3 (PCA3) and prostate-specific antigen (PSA) mRNAs, and total serum prostate-specific antigen (PSA) levels between prostate cancer (PCa), prostatic nodular hyperplasia (PNH), and control groups.

Variable	PCa (N = 22)[b]	PNH (N = 26)[c]	Control (N = 11)	P
PCA3	0.74 ± 1.48	0.31 ± 0.46	-	0.587
PSA	0.36 ± 0.40	0.20 ± 0.25	-	0.215
PCA3/PSA	6.08 ± 9.19	2.90 ± 3.02	-	0.535
Total PSA[a]	7.620 (4.64)	6.800 (7.98)	4.735 (5.87)	0.065

Means ± SD, Student *t*-test. [a]Median (interquartile range), Kruskal-Wallis. [b]N = 16 for *PCA3* and PCA3/PSA ratio. [c]N = 4 for *PCA3* and PCA3/PSA ratio.

PCA3 and *PSA* gene expression variables were categorized as presence (quantifiable expression) or absence (no quantifiable expression), and the frequency was also calculated. Additionally, the total serum PSA was categorized as PSA < 4 or PSA ≥4 ng/mL (reference value) (Table 4). The PCa group showed a higher frequency of *PCA3* and *PSA* detectable expression compared with the PNH group, while the PNH group also showed higher frequency of quantifiable expression compared with the control group. A higher frequency of individuals with levels of total serum PSA ≥ 4 ng/mL was also observed in the PNH and PCa groups compared with the controls.

Table 4. Frequency of prostate cancer antigen 3 (PCA3), prostate-specific antigen (PSA) (expression), and total serum PSA variables categorized in groups.

Presence	PCa (N = 22)	PNH (N = 26)	Control (N = 11)	P
PCA3	16 (72.7%)	4 (15.4%)	0	<0.001*
PSA	16 (72.7%)[b]	7 (26.9%)	1 (9.1%)[a]	<0.001*
PSA ≥ 4 ng/mL	20 (90.9%)	23 (88.5%)	6 (54.5%)[a]	0.020*

PCa = prostate cancer; PNH = prostatic hyperplasia. [a]Less frequent. [b]Most frequent by residue test. *Statistically significant.

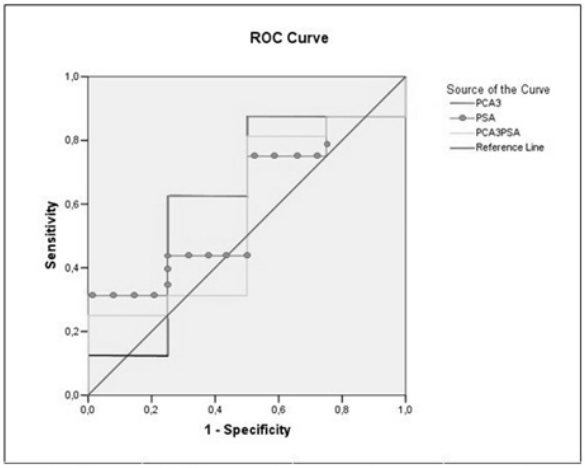
Performance of the markers as diagnostic tests

The assessment of sensitivity and specificity of the expression levels of *PCA3* and *PSA* genes, and the PCA3/PSA ratio as tests for differential diagnosis between PCa and PNH were performed using a ROC curve (Figure 1). No comparison with the control group was performed since there was no significant expression of the genes in this group.

According to the classification proposed by Mota and Filho (2009), values of the area under the curve (AUC) of between 0.60 and 0.70 are considered poor tests for diagnosis, while values between 0.50 to 0.60 are considered bad. Therefore, the expression of the *PCA3* gene can be considered poor for distinguishing patients with PCa and PNH. The *PSA* expression values and the PCA3/PSA ratio are bad for this comparison.

The sensitivity and specificity of total serum PSA in discriminating patients with PCa and PNH were also assessed by ROC curve (Figure 2). The levels of total serum PSA were also bad for distinguishing patients with PCa and PNH, with low sensitivity and specificity.

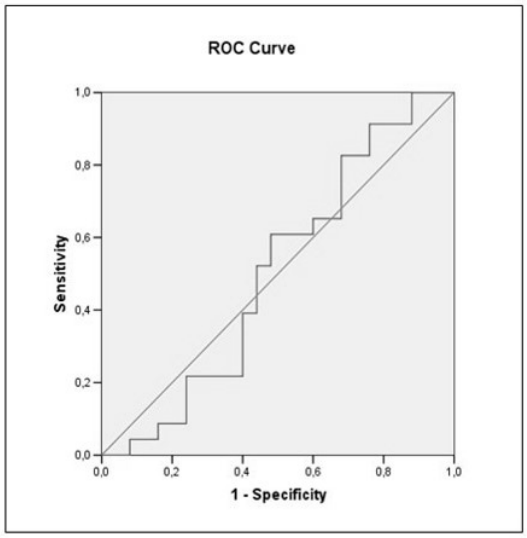
Cutoffs for which the test showed higher sensitivity and specificity for the detection of PCa and the frequency of individuals with values above and under the cutoff were determined for each variable (Tables 5 and 6). We observed a significant difference between the number of individuals with values above the cutoff in the PCa group for *PCA3* gene expression and PCA3/PSA ratio compared with the PNH group (P < 0.001). For the *PSA* gene expression and serum PSA level variables, no difference between the two groups was observed.



Variable	Area under curve	CI[a]	p
PCA3	0.625	0.290 – 0.960	0.450
PSA	0.596	0.349 – 0.843	0.449
PCA3/PSA	0.559	0.229 – 0.888	0.720

(a) Confidence interval at 95%

Figure 1. Receiver operating characteristic (ROC) curve for prostate cancer antigen 3 (*PCA3*) and prostate-specific antigen (*PSA*) gene expression and PCA3/PSA ratio.



Variable	Area under curve	CI[a]	p
Total PSA	0.503	0.336 – 0.670	0.975

(a) Confidence interval at 95%

Figure 2. Receiver operating characteristic (ROC) curve for total serum prostate-specific antigen (PSA).

Table 5. Values of cutoff determined for the variables studied.

Variable	Cutoff	Sensitivity	1-Specificity
PCA3	0.2219	0.625	0.250
PSA	0.9615	0.706	0.375
PCA3/PSA	0.5007	0.824	0.500
Total PSA	7.020	0.609	0.480

PCA3 = prostate cancer antigen 3; PSA = prostate-specific antigen.

Table 6. Frequency of individuals with higher and lower values than the cutoff for each variable considering only individuals with prostate cancer (PCa) and prostatic nodular hyperplasia (PNH).

	Variable	PCa	PNH	P
PCA3 (N = 20)**	<0.2219	6 (37.5%)	3 (75.0%)	<0.001*
	≥0.2219	10 (62.5%)	1 (25.0%)	
PSA (N = 23)**	<0.9615	14 (87.5%)	6 (85.7%)	0.834
	≥0.9615	2 (12.5%)	1 (14.3%)	
PCA3/PSA (N = 20)**	<0.5007	3 (18.8%)	2 (50.0%)	<0.001*
	≥0.5007	13 (81.2%)	2 (50.0%)	
Total PSA (N = 48)**	<7.020	9 (41.0%)	13 (50.0%)	0.256
	≥7.020	13 (59.0%)	13 (50.0%)	

PCA3 = prostate cancer antigen 3; PSA = prostate-specific antigen. *Statistically significant. **N = number of individuals in which the variable was detected. Fisher's exact test or chi-square.

A new analysis of total serum PSA was performed considering the ability of the marker to distinguish the control group versus the other two groups (PCa and PNH) (Figure 3). The AUC obtained for total serum PSA (0.727) indicates that this marker shows regular ability to predict the presence of any prostate alteration, either PNH or PCa.

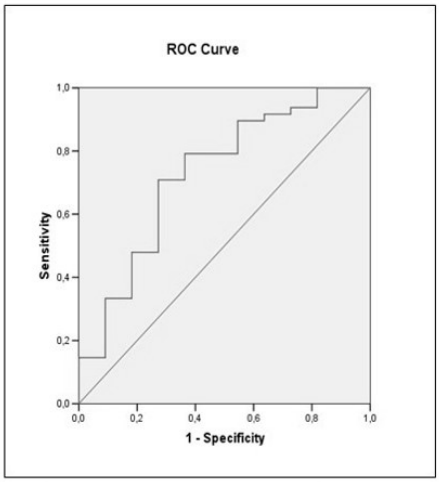


Figure 3. Receiver operating characteristic (ROC) curve for total serum prostate-specific antigen (PSA) considering the control group versus the prostate cancer (PCa) + prostatic nodular hyperplasia (PNH) groups.

The best cutoff for total serum PSA that showed higher sensitivity and specificity for diagnosing PCa or PNH, distinguishing them from patients with no alterations (control), was 5.560, with sensitivity = 0.708 and 1-specificity = 0.273. We determined the number frequency of subjects with total PSA levels above and below the cutoff and we observed more individuals with higher values in the PNH + PCa group compared with the control group (Table 7).

Table 7. Number of subjects with values higher and lower than the cutoff for total prostate-specific antigen (PSA) between the two groups.

	Variable	PCa + PNH	Control	P
Total PSA (N = 59)**	≥5.560	33	3	0.009*
	<5.560	15	8	

PCa = prostate cancer; PNH = prostatic nodular hyperplasia. *Statistically significant. **N = number of individuals in which the variable was detected. Fisher's exact test.

Besides the ROC curve, we conducted a new analysis comparing the frequency of PCa diagnosis by biopsy and the frequency of cases with detection of *PCA3* gene expression, independently of their values, and we obtained sensitivity and specificity values of 73 and 85%, respectively. The same analysis compared with biopsy was performed for detection of the *PCA3* gene expression and PCA3/PSA ratio, considering the test positive for individuals with expression levels greater or equal to the established cutoff (Table 5). As a result, we found a sensitivity of 62.5% and a specificity of 75.0% for *PCA3*, and 81.25 and 50.0% for the PCA3/PSA ratio, respectively.

Evaluation of markers according to the tumor classification

We performed analyses of the markers *PCA3* and *PSA* gene expression, PCA3/PSA ratio, and total serum PSA levels in patients diagnosed with PCa according to the Gleason score obtained by biopsy: a) Gleason score less than 7 (better prognosis); b) Gleason score greater than or equal to 7 (worst prognosis) (Table 8). Although the results did not show significant differences between the mean or median values, we observed a higher PCA3/PSA ratio in individuals with a poor prognosis, i.e., a Gleason score $\geq$ 7.

Table 8. Expression of prostate cancer antigen 3 (*PCA3*) and prostate-specific antigen (*PSA*) genes, PCA3/PSA ratio, and total serum prostate-specific antigen (PSA) according to the Gleason classification.

Variable	Gleason < 7 (N = 9)	Gleason ≥ 7 (N = 13)	P
PCA3	0.62 ± 0.69	0.81 ± 1.84	0.815
PSA	0.42 ± 0.41	0.33 ± 0.40	0.670
PCA3/ PSA	1.72 ± 1.77	8.70 ± 10.89	0.116
Total PSA[a]	8.20 (4.40)	7.29 (3.66)	0.453

Means ± SD, Student *t*-test; [a]Median and interquartile range, Mann-Whitney.

DISCUSSION

Although the PCA3/PSA ratio was higher among patients with PCa, the difference between groups (PCa and PNH) was not significant. No difference was found for expression levels of *PCA3* and *PSA* genes alone, although for *PCA3* the mean was higher in the PCa group. However, some studies have demonstrated significant differences between the expression levels of both genes

in the urine of patients with PNH and PCa following prostatic massage (Hessels et al., 2003; Groskopf et al., 2006; Haese et al., 2008; Neves et al., 2008; Mearini et al., 2009; Jamaspishvili et al., 2011). Possibly this significance was not found in our study because these variables presented large dispersion and variability, which are reflected in the magnitude of the standard deviation.

In the PNH group, four patients showed quantification for *PCA3* gene expression. Since studies have shown that *PCA3* expression is tissue-specific for the tumor, we did not expect to detect *PCA3* expression in this group (Bussemakers et al., 1999; Hessels et al., 2003). Considering that the classification in groups was based on the biopsy results (the gold standard), we cannot discard limitations in the technique, or the possibility that these PNH patients presented undetectable early PCa, since the sensitivity of biopsy varies and depends on the number of fragments analyzed. Among the patients with PCa, six showed no detectable expression of *PCA3* and *PSA* genes. This indicates that during the sample collection and prostatic massage there was insufficient release of prostate cells, or degradation may have occurred, which is common with urine samples owing to cell lysis. In the PNH group, *PSA* expression was detected in seven individuals, which was fewer than we expected. However, the prostate tissues in PNH flake less easily than those affected by the tumor, even after prostatic massage, owing to the increased cellular proliferation observed in PCa, which would explain these findings (Nelson et al., 2004). Among the control subjects, none showed *PCA3* gene expression and only one individual showed PSA expression, confirming the specificity of *PCA3* gene expression to detect prostate alterations.

The advantage of using urine for PCa screening is that it is a non-invasive method, but it requires care and is subject to variations associated with the prostatic massage performed by the physician. Furthermore, the study of gene expression in urine is a difficult procedure and requires fast handling and special care to avoid degradation. Urine is a material with many contaminants and PCR inhibitors that may interfere with the analysis and hinder the standardization of a preferred test (Toye et al., 1998).

No difference was found for total serum PSA, although the levels were higher in the PCa group. Several studies have discussed the limitations of PSA as a marker for PCa screening (Thompson et al., 2004; Hessels and Schalken, 2009; Dall'Oglio et al., 2011). Hessels et al. (2009) demonstrated that patients with serum PSA levels between 3 and 10 ng/mL have a 60-75% chance of presenting negative biopsies, while Thompson et al. (2004) showed that cancer can be found in men with PSA levels less than 4 ng/mL.

When we conducted the groups' categorization according to the presence or absence of gene expression, there was a higher frequency of *PCA3* detection in individuals with PCa compared with the PNH group. The same was observed for PSA. Therefore, the qualitative analysis of *PCA3* and *PSA* genes was more reliable than the quantitative analysis for distinguishing between PCa and PNH patients.

Since the lack of expression of the *PCA3* gene obviates the need for prostate biopsy, this marker is an important parameter for avoiding unnecessary biopsies (Haese et al., 2008; Gittelman et al., 2013). According to our results, *PCA3* gene expression indicates an 80% chance of positive biopsy, since among these individuals 16 had positive biopsies and only 4 had negative biopsies.

We also categorized the total serum PSA in individuals according to those with less than 4 ng/mL and those with ≥ 4 ng/mL PSA. There was a higher frequency of subjects with total PSA ≥ 4 ng/mL in the PCa and PNH groups compared with the controls. However, no difference between the PCa and PNH groups was observed, which corroborates that the total serum PSA does not present good specificity in screening for PCa, since it cannot differentiate between patients with

malignant alterations and those with benign alterations in all cases (Riegman et al., 1991; Balk et al., 2003; Riffenburgh and Amling, 2003; Thompson et al., 2004; Hessels and Schalken, 2009).

The AUC obtained from the ROC curve showed that quantitative analysis of *PCA3* gene expression is poor for differentiating between patients with PCa and those with PNH, according to the findings of Marks et al. (2007) and Jamaspishvili et al. (2011), who reported 0.678 and 0.671, respectively (Marks et al., 2007; Jamaspishvili et al., 2011). These values are considered poor but the method is still better than simply analyzing the *PSA* expression and ratio PCA3/PSA. However, Hessels et al. (2003) and Tinzl et al. (2004) reported AUCs for *PCA3* gene expression in urine of 0.717 and 0.87, respectively - values considered good for a diagnostic test for cancer (Hessels et al., 2003; Tinzl et al., 2004). These contradictory results could be due to the different sample sizes and methodologies used to quantify gene expression among the studies.

When cutoff values for each variable with higher sensitivity and specificity were estimated, we found that a frequency value equal to or greater than 0.2219 (expression level) for the *PCA3* gene was higher in the PCa group compared with the PNH group. Likewise, values equal to or greater than 0.5007 for PCA3/PSA ratios were higher in the cancer group. Thus, these results indicate that quantitative values of PCA3 expression and PCA3/PSA ratio could be used in clinical practice, but these values must be established in a larger group that is representative of the population in which the test will be applied. The cutoff value suggested by Hessels et al. (2003) was 0.200 for *PCA3* scores comparing urinary sediments in men with malignant changes and men with benign changes. Tinzl et al. (2004) estimated a cutoff of 0.500 for PCA3/PSA ratio in a prospective study of patients referred for detection of PCa - a value close to that observed in the present study (Hessels et al., 2003; Tinzl et al., 2004).

The analysis of total serum PSA according to the ROC curve showed a strong ability to detect any prostatic change compared with the control group, but without differentiating between benign and malignant changes. It is known that PSA should be evaluated individually for each patient according to the clinical condition and additional examinations, and should not be used alone for a complete clinical diagnosis owing to its low specificity (Riegman et al., 1991; Balk et al., 2003; Riffenburgh and Amling, 2003; Thompson et al., 2004; Hessels and Schalken, 2009).

The cutoff values estimated for the total serum PSA to distinguish between the two prostate changes (7.020 ng/mL), or to differentiate between any prostate change and the controls (5.560 ng/mL), are even higher than the reference value currently used (4 ng/mL). These findings confirm that serum PSA levels present low specificity in PCa screening (Riegman et al., 1991; Balk et al., 2003; Riffenburgh and Amling, 2003; Thompson et al., 2004; Hessels and Schalken, 2009) since we identified individuals with serum PSA values up to 7.020 ng/mL with no malignant prostatic changes.

Comparing the detection of PCa using the *PCA3* gene expression test and prostate biopsy, which is the gold standard for this study, we note that the sensitivity and specificity were 73 and 85%, respectively. The test proved to be more specific than sensitive, which is desirable for a test that eliminates the chance of a false positive result, avoiding repetitive biopsies.

Considering sensitivity and specificity for *PCA3* gene expression and PCA3/PSA ratio detection according to the estimated cutoff, it was observed that the PCA3/PSA ratio was more sensitive for differentiating between patients with PCa and those with PNH, while screening using only the *PCA3* test was more specific. Hessels et al. (2003) obtained a sensitivity of 83% and a specificity of 67% for the PCA3/PSA ratio with a cutoff of 0.200 (Hessels et al., 2003). Tinzl et al. (2004), with a cutoff of 0.500 for the PCA3/PSA ratio, obtained a sensitivity of 82% and a specificity of 76% (Tinzl et al., 2004).

There was no significant difference between the groups regarding the Gleason classifica-

tion and the biomarkers, but we observed a higher PCA3/PSA ratio in individuals diagnosed with high-risk cancer. The results suggest that there may be a positive association between the level of *PCA3* gene expression and worst disease prognosis. Jamaspishvili et al. (2011) performed the same analysis and found no difference between patients with high Gleason score compared with individuals with a Gleason score of less than 7. For total PSA, the authors observed significantly higher levels among individuals with advanced-stage tumor (Jamaspishvili et al., 2011).

Our results indicate the clinical usefulness of the combination of *PCA3* and *PSA* as a molecular marker in the early diagnosis of prostate cancer, and the need for combining as many analytical data as possible with the clinical and demographic data to achieve the maximum level of diagnostic accuracy.

CONCLUSION

Despite initial studies involving the *PCA3* gene, its clinical utility as a marker is still uncertain. According to this study, patients without prostate changes have no measurable expression of the *PCA3* gene in the urine, but the test was not able to exclude completely patients with PNH. The results show the need to analyze a larger number of samples in order to establish a score that can predict a positive biopsy.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Instituto Hermes Pardini. K. Braga Gomes is grateful for the CNPq Research Fellowship (PQ).

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