RESEARCH ARTICLE

Evaluation of Combined Quantification of PCA3 and AMACR Gene Expression for Molecular Diagnosis of Prostate Cancer in Moroccan Patients by RT-qPCR

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Abstract

Prostate cancer (PCa) remains one of the most widespread and perplexing of all human malignancies. Assessment of gene expression is thought to have an important impact on cancer diagnosis, prognosis and therapeutic decisions. In this context, we explored combined expression of PCa related target genes AMACR and PCA3 in 126 formalin fixed paraffin embedded prostate tissues (FFPE) from Moroccan patients, using quantitative real time reverse transcription-PCR (RT-qPCR). This quantification required data normalization accomplished using stably expressed reference genes (RGs). A panel of twelve RG was assessed, data being analyzed using GenEx V6 based on geNorm, NormFinder and statistical methods. Accordingly, the hnRNP A1 gene was identified and selected as the most stably expressed RG for reliable and accurate gene expression quantification in prostate tissues. The ratios of both PCA3 and AMACR gene expression relative to that of the hnRNP A1 gene were calculated and the performance of each target gene for PCa diagnosis was evaluated using receiver-operating characteristics. PCA3 and AMACR mRNA quantification based on RT-qPCR may prove useful in PCa diagnosis. Of particular interesting, combining PCA3 and AMACR quantification improved PCa prediction by increasing sensitivity with retention of good specificity.

Keywords: Prostate cancer- reference genes- first biopsies- molecular diagnostics- PCA3 and AMACR ratio- threshold

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Introduction

Prostate cancer (PCa) is one of the most common causes of cancer worldwide affecting men. PCa diagnosisis mainly based on determination of serum prostate specific antigen (PSA) in combination with digital rectal exam (DRE) (Smith et al., 1996). Nevertheless, PSAstill remains a poor predictor of PCa because of the lack of both sensitivity (Se) and specificity (Sp) of the test (Kilpeläinen et al., 2010).

For this reason, elaborating a new strategy in PCa screening based on molecular tools represents an urgent requirement. Indeed, gene expression has emerged as an important tool for PCa diagnosis, prognosis and therapeutic care. In this context, a number of biomarkers have been identified and proved their relevance in PCa diagnosis. One of the most described biomarkers is the Prostate Cancer Antigen 3 (PCA3), which is expressed only in prostate tissues, and overexpressed in PCa. Since PCA3is a non-coding RNA, many assays based specificallyon RNA detection (Northern blot and TMA) analysis were developed (Bussemakers et al., 1999). The first experiments were performed on tissue samples but several studies were conducted to explore PCA3 expression in other biological samples such as blood or urine (Dijkstra et al., 2014). In line of this, a commercial kit for PCa diagnosis called Progensa® PCA3 assay was developed (GenProbe). The assay employs PSA mRNA as an internal control to normalize for the amount of PCA3mRNA in urine samples (Groskopf et al., 2006). The diagnostic performance of the PCA3 test has been extensively studied as it has beenapproved bytheFood and Drug Administration (FDA) as an aid tool for decision making in the repeat biopsy setting (Rittenhouse et al., 2013), with a cut-off value of 25. Thus, PCA3 represents a promising screening biomarker that will likely require its utilization with other biomarkers to strengthen the diagnosis (Ouyang et al., 2009; Auprich et al., 2011).

In the last yearsα-Methylacyl-CoaRacemase (AMACR) starts to be considered as a biomarker that has proven its efficiency as a PCa predictor (Rubin et al., 2002). Many variants of AMACR transcripts were

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generated by alternative splicing from a single gene locus (Mubiru et al., 2004). AMACR is nowadays routinely used in immunohistochemistry testing. Indeed, it has been demonstrated that AMACR allowsthe distinction between cancer and benign prostate pathologies with high Se and Sp for prostatic carcinoma (Magi-Galluzzi et al., 2003). Moreover,it has been shown that AMACRis a highly specific marker for PCa cells, even in the earliest stages of malignant progression (Ouyang et al., 2009).

Quantitative real-time polymerase chain reaction (qPCR) is a method of choice, often used to identify genes that contribute to cancer prognosis and management (de Kok et al., 2002; Span et al., 2002). qPCR is a rapid, reliable and accurate technique which can sensitively and specifically quantify DNA as well as mRNA following reverse transcription (RT-qPCR) (Lie and Petropoulos, 1998; Bustin and Nolan, 2004). However, the RT-qPCR based quantitative gene expression measurements in different samples require data normalization to control for non-biological variation introduced during sample preparation such as differences in cellular input, RNA quality, and RT efficiency between samples (Huggett et al., 2005). These problems could be overcome using a stable reference gene (RG) for gene expression quantification normalization.

Although many RGs selection studies were described in the literature, identification of the suitable RG with a stable expression in prostate tissues samples context has not been enough explored (Mearini et al., 2009; Kusuda et al., 2013).

In the present study, the utility of quantifying and profiling PCA3 and AMACR gene expression for PCa prediction and diagnosis was explored byRT-qPCR method using Biopsy samples from Moroccan patients. The gene expression quantification of both PCA3 and AMACR was normalized using hnRNP A1 gene (heterogeneous nuclear ribonucleoprotein A1) that was selected as the most stable from a panel oftwelve analyzed.

Materials and Methods

Cell lines and prostate tissues samples
Cells culture

Three cell lines purchased from the American Type Culture collection (ATCC, Manassas, VA, USA) were used, LNCaP (CRL-1740), VCaP (CRL-2876) and the Primary Prostate Epithelial Cells; Normal, Human (NPCs, PCS-440-010). The LNCaP and VCaP were cultured in Roswell Park Memorial Institute medium (RPMI1640) and Dulbecco's Modified Eagle's medium (DMEM) respectively, as described.

Tissues

The formalin fixed paraffin embedded prostate (FFPE) tissues samples were obtained from 126 patients (age range 52-73 years, PSA levels range 6.16-15.9 µg/l) All samples were provided by the pathology department at the Mohamed V military teaching hospital in Rabat, Morocco (HMIMV). From each patient a paraffin-embedded block was selected and 10 µm sections were used for further analysis.

All patients gave their informed consent and the study was approved by the Ethical Committee of the Faculty of Medicine at University Mohammed V, Rabat, Morocco.

RNA isolation and cDNA synthesis
RNA extraction

Total RNA from cell lines pellet was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's recommendations for cultured cells. Whereas for FFPE sections, the extraction has been performed using the PureLink™ FFPE Total RNA Isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

On the whole the isolated RNA concentration were measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), the purity and integrity of the RNA were assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The isolated RNAs were stored at -80°C until use.

cDNA synthesis

One microgram of RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Identification of optimal RGs

Twelve genes were selected to identify the most stably-expressed RG to be used in RT-qPCR studies of PCa. The RGs candidates were selected based on previous reports (Radonić et al., 2004; Ohl et al., 2006). As far as we know, all these genes are expressed constitutively in prostate tissues and have unrelated cellular functions and are not co-regulated.

In order to identify the best RG for PCa gene expression quantification, RT-qPCR was used for the generation of expression data. The obtained Ct values were used in GenExV6 Standard software (bioMCC, Freising, Germany) that combines two statistical analysis tools geNorm and Normfinder(Vandesompele et al., 2002).

Primers design

Primers were designed using both Primers3 (http://simgene.com/Primer3)and genscript(https://www.genscript.com/ssl-bin/app/primer) softwares.

In our study the primer sets designed for PCA3 gene amplification can detect all transcripts, and can hybridize either to the junction between exon1-exon3, exon1-exon2 or exon2-exon3.

For AMACR gene, since there are many variants of the gene (Mubiru et al., 2005), the specific primers were designed in such a way to recognize a common region to all variants.

The primers sequence are available upon request.
Real-time quantitative RT-PCR

PCR reaction was performed using the ABI/Fast 7500 Real-Time PCR System (Applied Biosystems) using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). The PCR mix contained 12.5 µL Universal Master Mix, 1.25 µL of Taq-Man Gene Expression Assay

primer-probe mixes (Applied Biosystems), 5 µL of cDNA (50 ng), DEPC water was used to achieve a final reaction volume of 25µL. Amplifications were performed starting with 95°C for 20 sec, 50 cycles at 95°C for 1 sec, and a combined annealing and extension step at 60°C for 30 sec.

Statistical analysis

Statistical analysis was performed using XLSTAT-Life software a Microsoft Excel add-in (http://www.xlstat.com). Mann–Whitney U-test was used to analyze the expression differences of candidates RGs between BPH and cancerous tissues in cell lines and patients samples. The obtained p-values <0.05 from Mann–Whitney U-test were considered statistically significant.

To evaluate the efficacy of PCA3 and AMACR mRNAs as specific biomarkers of PCa, data collected from the genes quantification ratios (Ct-RG/CT-target genes), were summarized in a Receiver Operating Characteristic curve. ROC corresponds to the graphical representation of the couple (1 – specificity, sensitivity) for the various possible threshold values (Perkins and Schisterman, 2006). The threshold was derived from the ROC curve and matches with an optimal combination between Se and Sp. The area under curve (AUC) which is an important parameter to determine the diagnostic value of the test was also calculated(Hanley and McNeil, 1982).

Results

Reference genes selection for gene expression quantification in PCa

In order to identify the most suitable RG for relative quantification of the gene expression in prostate cells,

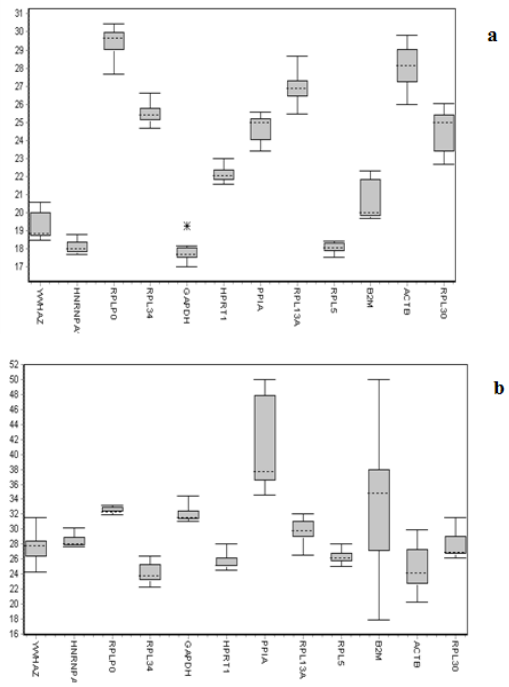

Figure 1. Cycle Threshold Value Distribution for the Different Analyzed Reference Genes (RG)
Ct values were calculated using qPCR for different RG candidates and they are showed as boxes representing lower and upper quartiles with medians in prostate cell lines (a) and in prostate biopsies (b).

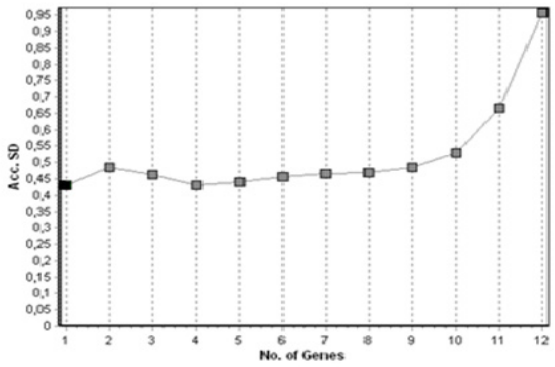

Figure 2. Determination of the Optimal Number of Reference Genes for Normalization.
Results are presented as per the output file of the geNorm software as Accumulated standard deviation (Acc.SD), the lowest value represented by the black dot indicate the optimal number of RGs recommended for normalization in prostate samples. While the X-axes represents the optimal number of genes required for normalization, the Y-axes Acc.SD values.

a panel of twelve gene candidates, commonly used in different cellular contexts as RG for quantification in the RT-qPCR, has been selected. This panel includes RPL30, B2M, ACTB, YWHAZ, RPL13, GAPDH, RPLP0,PPIA, RPL34, hnRNP A1, RPL5 and HPRT1. All these RGs have been already used, according to the literature, for the quantification normalization of different genes using RT-qPCR in different tissue contexts (Mane et al., 2008; Kheirelseid et al., 2010). The analysis of the selected RGs expression was first performed using the well-established human PCa and normal cell lines used routinely in our

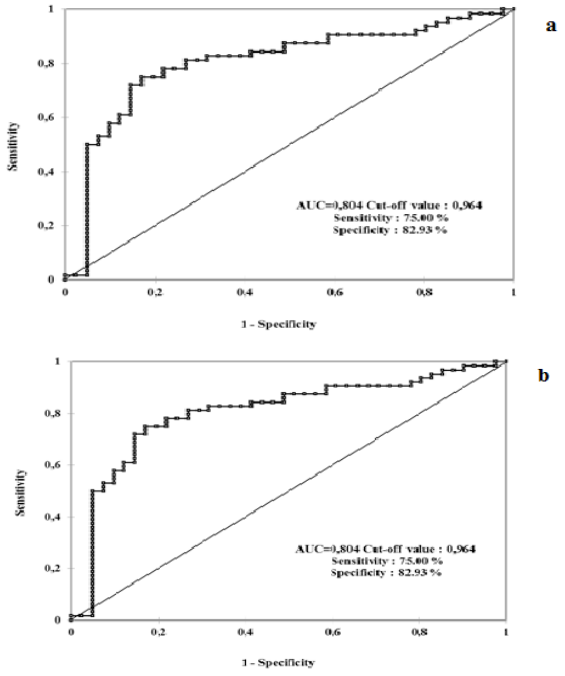

Figure 3. Sensitivity and Specificity of Target Genes mRNA Quantification for Detecting Prostate Cancer.
The receiver operating characteristic curve analysis is used to determine both sensitivity and specificity of each used genetic marker. ROC curve analysis of PCA3 expression in biopsies (a) and ROC curve analysis of AMACR expression in biopsies (b).

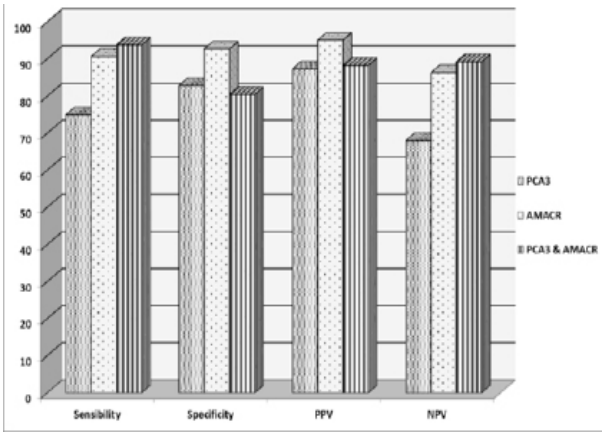

Figure 4. *PCA3* and *AMACR* Ratio and Clinical Relevance of Their Combination in PCa Diagnosis
Histogram comparing the sensitivity and specificity, positive predictive value and negative predictive value for PCA3 and AMACR and their combination in the PCa representing the performance of the PCA3 and AMACR and their combination in the prostate cancer diagnosis.

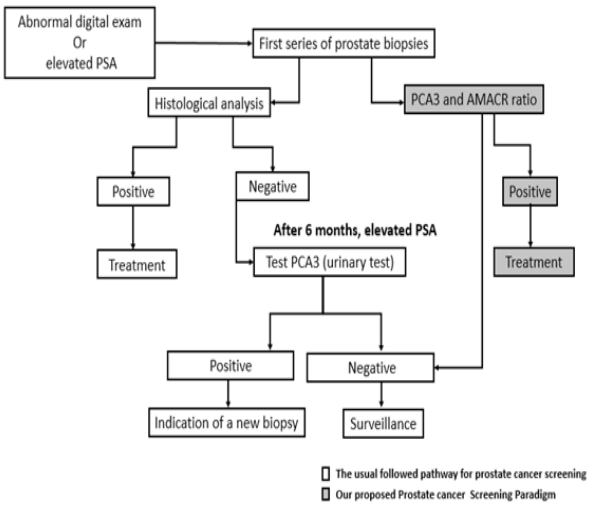

Figure 5. Schematic Representation of the Proposed Prostate Cancer Screening Paradigm.
The white square represents the usual followed pathway for the analysis and screening of the prostate cancer. The gray squares represent our proposed new pathway.

laboratory including LNCaP, VCaPandNPrEC. In order to evaluate the expression distribution of candidate RGs in normal and PCacell lines, we used non-parametric Mann-Whitney U-test as described in material and methods. For all the tested RGs there was no difference in gene expression between cancerous and normal cells lines except B2M gene which showed significant differences in the expression with ap-value equal to 0.036.Nevertheless, based on their Ct values, the analysis of the expression stability of these genes could not be accurately achieved (Figure 1a). Therefore, we used the well-established GenEx V6 Standard software (bioMCC, Freising, Germany) which combines two statistical analysis tools geNorm and NormFinder (Vandesompele et al., 2002). According to geNorm analysis HPRT1and hnRNP A1genes were the most stably expressed genes,in contrast B2M was the least stable gene (Table 1). In line with this, NormFinder analysis has also identified HPRT1 and hnRNP A1as the most stably expressed genes (Table 1) which indicates a high concordance between the two different softwares demonstrating the accuracy of the obtained results.

To extend our analysis for the most stableRG, the same analysis described above was performed for human prostate cell lines using this timehuman prostate FFPE biopsies. As described in Figure 1b, a fluctuation is observed in the Ct values obtained for the same gene for each biopsy sample used. The RPLP0, RPL5 and hnRNP A1showed the small range of Ct values variation, while B2M, ACTB and PPIA had the widest range of Ct values (Figure 1b). The significance of Ct variation between normal and cancerous prostate biopsies was checked. However, it is still difficult to determine which gene is the most stably expressed between the different analyzed samples using only the CT value. Therefore, we used the GeNorm software to measure the stability of these genes in the used biopsies. As indicated in Table 1, the genes

Table 1. The Ranking of Candidate RGs According to Their Expression Stability by GeNorm and NormFinder in Human Prostate Cancer Cell Lines and Biopsies

Human prostate cell lines				Prostate biopsies			
GeNorm	M-value	NormFinder	SD	GeNorm	M-value	NormFinder	SD
gene symbol	M	gene symbol	SD	gene symbol	M	gene symbol	SD
HNRNPA1	0.022	HPRT1	0.446	HNRNPA1	0.046	HNRNPA1	0.430
HPRT1	0.022	RPL5	0.447	RPL5	0.046	YWHAZ	0.869
RPL13A	0.037	HNRNPA1	0.476	HPRT1	0.049	HPRT1	0.996
PPIA	0.040	RPL34	0.571	RPL34	0.057	RPL5	1.022
ACTB	0.042	PPIA	0.613	RPL30	0.063	RPL13A	1.372
RPL5	0.045	GAPDH	0.726	RPLP0	0.067	GAPDH	1.623
RPL34	0.049	RPL13A	0.741	GAPDH	0.074	RPL34	1.782
RPLP0	0.052	YWHAZ	0.852	RPL13A	0.082	ACTB	1.832
GAPDH	0.056	RPLP0	0.950	YWHAZ	0.090	RPL30	2.258
RPL30	0.059	ACTB	1.044	ACTB	0.104	RPLP0	2.954
YWHAZ	0.063	B2M	1.097	PPIA	0.118	PPIA	5.092
B2M	0.066	RPL30	1.150	B2M	0.161	B2M	8.838

with the lowest M value (Vandesompele et al., 2002) were RPL5, hnRNP A1 and HPRT1indicating that these are the most stable genes and confirming therefore the results obtained for the PCa cell lines described above (Table 1). To strengthen our results, we use NormFinder program and calculate the stability of the studied genes. Table 1 describes the stability factor obtained for each gene in the different analyzed biopsy samples. Once again, the most stable genes identified by NormFinder software are RPL5, hnRNP A1 and HPRT1which have the lowest stability value (Table 1). This confirms the results obtained by geNorm program (Table 1).

Moreover; NormFinderalgorithm, allows the calculation of another factor called accumulated standard deviation (Acc.SD). Acc.SD indicates the optimal number of RGs theoretically required for a reliable normalization. The number of genes that give the lowest Acc.SD value will be the optimal RGs to be used for the normalization. For our analysis, the calculated Acc.SD suggests that one gene is enough for reliable normalization (Figure2).

Gene expression quantification of PCA3 and AMACR

As indicated above, HPRT1, hnRNP A1and RPL5were the most stable RGs in both cell lines and human biopsies samples (Figure 1 and 2).In addition, andaccording to the calculated Acc.SD, a single gene is sufficient for a reliable genetic quantification in prostate biopsies. Therefore, we decided to usehnRNP A1for an accurate quantification of both PCA3 and AMACR transcriptsin different biopsies samples, since the amplification efficiency ofhnRNP A1 transcripts was very close to the amplification efficiency of both PCA3 and AMACR transcripts (data not shown). We used 126 FFPE biopsy samples from which 21 samples were excluded due to the low expression of the RGhnRNP A1 (Ct value $\geq$ 35).Indeed, the amount of RNA extracted from the tested biopsy should be enough for an accurate sample analysis. Therefore only samples with hnRNP A1 Ct value inferior to 35 have been considered for the analysis.

Therefore 105 samples were available for the accurate quantification analysis. In these 105 samples, 64 samples were confirmed to be positive PCa and the rest 41samples, is non-cancerous prostate tissues. We used the ROC analysis to calculate the Sp, the Se and the cut off value of expression. As shown in Figure 3, a cut-off value for the PCA3 expression was determined to be 0.964 that gives rise to the best combination of Se and Sp of 75.0 % (95% CI: 62.60%-84.98%)and 82.9% (95% CI: 67.94%-92.85%) respectively and with a performance value AUC of 0.8 (95% CI: 0.716-0.892) (Figure 3a). Furthermore, we performed the same ROC analysis this time for AMACR gene expression quantification. A cut-off of 1.035 was determined with a Se and Sp of 90.62% (95% CI: 80.70%-96.48%) and 92.7% (95% CI: 80.08%-98.46%) corresponding to an AUC value of 0.94 (95% CI: 0.888-0.985) (Figure 3b). Moreover, while the positive predictive values (PPV) determined by the ROC analysis for bothPCA3 and AMACR gene expression evaluation were 87.3% (95% CI: 75.52%-94.73%) and 95.1% (95% CI: 86.29%-98.97%) respectively, the negative predictive value (NPV) were 86.4 % (95% CI: 72.65%-94.83%) and 68.0 % (95% CI: 53.30%-80.48%) respectively. In addition, we performed an analysis of ROC curve by combining this time the expression ofbothPCA3 and AMACR genes. In this analysis, a sample will be considered positive if it shows an overexpression of only one of these genes (above the cut-off)and will be considered negative if both genes are expressed normally (under the cut-off). The combination of these two biomarkers gave rise to a Se and Sp of 93.8% (95% CI: 84.76-98.27%), and 80.5% (95% CI: 65.13%-91.18%) respectively with a PPV of 88.2% (95% CI: 78.13%-94.78%) and a NPV of 89.2% (95% CI: 74.58%-96.97%) (Figure 4).

Discussion

In the last decade, PCa diagnosis has improved especially with the emergence of new biomarkers. However there's no single marker with no gaps that can definitely assert PCa diagnosis. PCA3 is the first non-coding RNA described in relation to PCa and proved to be exclusively expressed in the prostate tissues andoverexpressed specifically in PCas.We have used RT-qPCR-based relative quantification toevaluatethe expression of PCA3 and another genetic biomarker AMACR in both cancerous and normal prostate biopsies. Nevertheless, the relatively degraded nature of RNA isolated from FFPE tissue can lead to important variation in the detection of gene expression between samples, it was compulsory to select the most stable RGs that can counter this variation and make the PCA3 or other prostate RNA relative quantification more accurate. For this reason, we assessed the ability of 12 genes to be suitable references for the specific normalization of PCA3 and any other prostate gene transcripts in FFPE prostate biopsies. Two comparative ΔCt methods, GeNorm and NormFinder have been used for this analysis and both have identified the three RGs,RPL5, hnRNP A1and HPRT1 as the most stable genes in both prostate cell lines and FFPE tissues. To the best for our knowledge, this is the first systematic evaluation study of RG candidates for the specific normalization of prostate gene expression using RT-qPCR. Furthermore, according to the NormFinder analysis a single gene was sufficient for a reliable genetic quantification in prostate biopsies, therefore wedecided to use hnRNP A1to normalize this quantification since the amplification efficiency of hnRNP A1 transcripts was very close to the amplification efficiency of both studied prostates genes PCA3 and AMACR transcripts (data not shown). Recently Nadimintyet al., (2015) have shown that hnRNP A1 is slightly overexpressed in prostate cancer tissue compared to normal tissue. Nevertheless this expression has mainly been measured using western blotting analysis which is well established to be a no quantitative method. In addition, qPCR analysis of hnRNP A1 mRNA expression performed on ten samples has shown that overexpression is observed in only five analyzed samples (Nadiminty et al., 2015).

After the identification of hnRNP A1 as the mostsuitable RG, wefirst went to analyze the expression level of PCA3 in both cancerous and normal prostate cells using

RTqPCR. In this case, 105 prostate formalin-fixed paraffin embedded (FFPE) biopsy samples have been analyzed, 64 of which are confirmed PCawhereas 41arenormal prostate tissue. Using the ROC analysis we have been able to determine a cut-off of 0.964 that gives rise to the best Se and Sp of 75.00 % and 82.93 % respectively. Indeed, the combination of Se and Sp of our test was greater comparing to those described in previous works showing therefore that our test is highlyaccurate (Hessels and Schalken, 2009). As mentioned above, PCA3 test is advised in the case of an elevated level of PSA to decide whether or not a biopsy analysis has to be repeated (Ploussard et al., 2010). Therefore anaccurate test with a high degree of both Se and Sp is becoming a requirement. Although the PCA3 test is mainly performed with urine sample which has the advantage to be anon-invasive test, our test still remain of great importance since it could be used with the mandatory picked-up tissue samples of the first biopsy. This will not only allow the first pathological examination but also a PCA3 based molecular test to confirm whether or not another biopsy is required.

Another prostate biomarker that was demonstrated to allow distinction between cancer and benign prostate pathologies and was shown to be a useful immunohistochemically marker for PCais AMACR (Magi-Galluzzi et al., 2003). As it was the case for PCA3, we also analyzedusing RTqPCR the expression level of AMACR in the same patient samples used for PCA3 analysis. The ROC analysis has generated a cut-off of 1.035 with a Se and Sp of 90.62 % and 92.68 %. Once again, and compared to previous work, our AMACRbased test is one of the best in terms of Se and Sp and therefore could be used to accurately distinguish between benign and malign prostate tissues (Jiang et al., 2001). AMACR is a well-established cancer prostate marker that is detected using immunohistochemistry to distinguish between malign and benign prostate tissues (Molinié et al., 2004; Herawi and Epstein, 2007). In addition,the role of AMACRoverexpression in triggering PCa is well documented (Rubin et al., 2002; Zheng et al., 2002). This makes the quantification of AMACR gene expression an attractive tool in order to enhance PCa molecular diagnosis. Nevertheless the use of a single marker still remains insufficient for a test to be highly specific. For this reason we decided to analyze both the Se and Sp of our test by combining the quantification of both factors, PCA3 and AMACR. In this analysis, a sample will be considered positive if it shows an overexpression of only one of these genes (above the cut-off) and will be considered negative if both genes are expressed normally (under the cut-off). Indeed, the combination of these two biomarkers increased both the Se and Sp of our test and therefore demonstratesthat using the two biomarkers in a single test will increase the performance of the prostate molecular diagnosis and consequently makes the biopsy decision highly accurate.

In conclusion, our results demonstrate that the combined quantification of PCA3 and AMACR genes expression using RT-qPCR is indeed a promising marker for PCamolecular diagnosis and increase the predictive accuracy of the biopsies outcome (Figure 5).

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