Characterization of Kallireins and microRNAs in Urine Sediment for the Discrimination of Prostate Cancer from Benign Prostatic Hyperplasia

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Introduction

Prostate cancer (PCa) is the most common cancer in European elderly men. Over 200 cases every 100.000 subjects are diagnosed in Northern and Western Europe and a continuous increase is observed in Eastern and Southern Europe [1]. Similarly, Benign Prostatic Hyperplasia (BPH) is a condition closely related to ageing [2] and consists of a benign enlargement of prostatic adenoma size that gives rise to Lower Urinary Tract Symptoms (LUTS). These urinary disorders can occur in up to 30% of men older than 65 years [3]. These two conditions are frequently coexisting, and up to 20% of BPH patients subjected to trans urethral resection of prostate are incidentally found to harbour PCa [4]. PCa and BPH also share relevant features at the genetic level [5] and inflammation has been suggested to play an important role in both urologic conditions [6]. Nonetheless, they take place in different anatomic sites [7] and BPH does not affect the risk of developing PCa [8]. The molecular mechanisms responsible for benign or malignant growth of prostate epithelial cells are partially unknown, although cellular overgrowth is a common event. The measurement of serum PSA together with digital rectal examination (DRE) represents the primary diagnostic tool to suspect PCa, whereas definitive diagnosis is achieved by prostate biopsy [9]. In young men, serum PSA levels < 2–3 ng/mL are considered normal: beyond this value, higher levels of PSA are associated with higher risk of having PCa [9]. Nevertheless, poorly differentiated forms of PCa could have low serum levels of PSA as well as high serum PSA levels could be due to benign conditions including BPH [10]. The low specificity of PSA, which is a prostate specific and not a cancer specific marker, is the cause of many unnecessary biopsies. On the other hand, the detection rate of baseline biopsy is only 25-38% [11,12], whereas false-negative amounts to about 23% [13]. When PSA levels remain elevated, rebiopsy is performed and PCs can be detected in 7-22% of cases at the first rebiopsy and 2-10% at the second one [14-16]. Moreover, this maneuver is affected by bleeding, urinary infection risk and pain and discomfort for the patient. Thus, the high false positive rate of PSA together with the low detection rate of biopsy convict the patient to a quite often unnecessary and uncomfortable clinical itinerary. Nevertheless these limitations, prostate biopsy remains the standard tool to confirm the diagnosis
of PCA. These many limitations of current diagnostic management of PCA demonstrate the urgent need for new and more accurate tools and methodologies to detect cancer. KLK3 is the gene coding PSA and belongs to the family of kallikreins (KLKs), which accounts for 15 highly conserved serin proteases which have been demonstrated to be involved in hormone-dependent diseases including PCA [17,18]. The scientific interest towards kallikreins in cancer biomarker discovery is justified by their alterations in expression in several cancers which was demonstrated at the protein level [19-21]. KLK11 proved to be altered in PCA at the protein level [22], whereas KLK13 has been described as a favorable prognostic marker of ovarian cancer [23], and shares 51% amino acid identity with KLK11. PCA3 is a new biomarker which is detectable in post DRE urine sediments. Its diagnostic accuracy for PCA is higher than total PSA in men with elevated PSA levels. Though many studies have demonstrated the ability of PCA3 to detect PCA, its employment in clinical routine is under debate [24].

There is a growing scientific interest in microRNAs as markers for PCA; these small RNAs preside over many cellular functions and regulate gene expression [25] and their alteration has been observed in cancer [26]. The first profiling of miRNA involved in PCA dates back in 2007 [27]; from that point, many progresses have been made towards the characterization of different sets of miRNAs for PCA diagnosis and prognosis [28]. Many studies have demonstrated the modulation of miR-9 in various cancers, while miR-19a-3p belongs to the oncogenic miR-17-19 cluster, also named oncomiR-1 [29]. Both miR-9-3p and miR-19a-3p were evaluated in urine sediments and comparisons were made between PCa and BPH samples before prostate biopsy and after attentive DRE. Briefly, a firm pressure on the gland from base to apex and from lateral to median side were made between PCa and BPH. mRNA and miRNA were evaluated in urine sediments and comparisons were made between PCa and BPH. A brief description of the study is given in Table 1. The expression values of KLK3, KLK11, KLK13, miR-9-3p and miR-19a-3p were evaluated in urine sediments and comparisons were made between PCa and BPH. Logistic regression analysis was performed with Taqman probes (Applied Biosystems, Foster City, CA) on a BioRad iCycler (BioRad, Hercules, CA). The probe sequences used are listed in Table 2. In a total PCR reaction volume of 20 µL, 4 µL of diluted cDNA (1:20) were added. Each sample was run in triplicate and results were averaged. In parallel, negative controls without template were analyzed. The -ΔCt method was used to calculate the

**Materials and Methods**

**Patients**

Patients - From June 2012 to April 2013, 41 Caucasian subjects affected by PCA and 38 age-matched subjects affected by BPH were enrolled. Ethics Committee of the University of Perugia approved the study protocols. All subjects provided written informed consent. PCA group included patients with localized PCA and age < 75 years. Robot assisted radical prostatectomy was performed in all included PCA patients. For PCA group exclusion criteria were age > 75 years, locally advanced or metastatic PCA. BPH group included subjects with negative needle biopsy, PSA levels higher than 3 ng/ml which underwent transurethral prostate resection with confirmed diagnosis of BPH. Exclusion criteria for BPH group were urinary infections, bladder stones, catheterization, age > 75 years. The clinical parameters of all subjects are reported in Table 1. The expression values of KLK3, KLK11, KLK13, miR-9-3p and miR-19a-3p were evaluated in urine sediments and comparisons were made between PCa and BPH. Logistic regression analysis was performed to estimate the diagnostic accuracy of markers under analysis in predicting PCA.

**Sample processing**

**Urine collection:** First catch voided urine was collected in sterile cups before prostate biopsy and after attentive DRE. Briefly, a firm pressure on the gland from base to apex and from lateral to median side with three stokes for each lobe was performed. Urine was stored at 4°C and processed within 4 hours. After centrifugation (2000 × g, 10 min, 4°C) cell pellets were washed twice with 1X calcium-free phosphate buffered saline. Cell lysates were obtained adding 300 µL Lysis and Stabilization Buffer (Total RNA Extraction Kit, Norgen Biotek Corp. Ontario, Canada).

**RNA extraction:** Total RNA, including miRNAs, was extracted from cell lysates with Total RNA Extraction Kit (Norgen Biotek Corp. Ontario, Canada) following the manufacturer’s recommendations. RNA has been subjected to qualitative and quantitative assessments through Bioanalyzer, Nanodrop and Qubit RNA HS Assay Kit (Life technologies). RNA was stored at -80°C until use.

**Reverse transcription and real-time PCR**

mRNAs: 15 ng of total RNA were reverse transcribed with SuperScript VILO cDNA Synthesis Kit (Invitrogen, Life Technologies, CA, USA) according to manufacturer’s protocol. Real-time PCR assays were performed with Taqman probes (Applied Biosystems, Foster City, CA) on a BioRad iCycler (BioRad, Hercules, CA). The probe sequences used are listed in Table 2. In a total PCR reaction volume of 20 µL, 4 µL of diluted cDNA (1:20) were added. Each sample was run in triplicate and results were averaged. In parallel, negative controls without template were analyzed. The -ΔCt method was used to calculate the

**Clinical parameters (total subjects: 79)**

| Parameter          | PCa (41) | BPH (38) |
|--------------------|----------|----------|
| Median Age         | 65 (54-75) | 66 (48-74) |
| Median PSA (ng/mL) | 7.32 (3.54-50) | 7.24 (1.38-21.22) |
| **Clinical Gleason Score** | | |
| 6 (3+3)            | 32       |
| 7 (3+4)            | 6        |
| 7 (4+3)            | 1        |
| 8 (4+4)            | 1        |
| 9 (5+4)            | 1        |
| **Clinical staging** | | |
| ctT1c              | 23       |
| ctT2a              | 13       |
| ctT2c              | 3        |
| ctT3a              | 2        |
| **Pathological Gleason Score** | | |
| 6 (3+3)            | 29       |
| 7 (3+4)            | 9        |
| 7 (4+3)            | 1        |
| 8 (4+4)            | 1        |
| 10 (5+5)           | 1        |
| **Pathological Staging** | | |
| pT2a               | 4        |
| pT2c               | 28       |
| pT3a               | 6        |
| pT3b               | 3        |

| Positive Surgical Margin | 15% | |

**Table 1:** Clinical parameters of subjects included in the study.

| Gene      | Name                            | Sequence          | Exons connected | Amplicon |
|-----------|---------------------------------|-------------------|-----------------|----------|
| KLK3      | kallikrein-related peptidase 3 | Hs02576345_m1     | 1-2             | 83 bp    |
| KLK11     | kallikrein-related peptidase 11| Hs01100849_m1     | 4-5             | 77 bp    |
| KLK13     | kallikrein-related peptidase 13| Hs01087307_m1     | 4-5             | 82 bp    |
| PCA3      | Prostate cancer antigen 3      | Hs01371938_m1     | 2-3             | 80 bp    |
| ACTB      | Actin beta                      | Hs0106665_g1      | 2-3             | 63 bp    |

**Table 2:** TaqMan Probe sequences used for mRNA analysis.
Results

Qualitative and quantitative assessment of RNA

Total recovery of RNA was not significantly different among samples. Mean RNA integrity number (RIN) was 8.7 (8.3-9.0) and 260/280 ratio was 1.84 (1.84-2.03). Minimal variations in total RNA content were corrected during reverse transcription using fixed RNA input (15 ng total RNA for KLK3, KLK11, KLK13; 7.5 ng for miR-9-3p and miR-19a-3p). Relative quantification of real-time PCR data was performed referring the levels of selected targets to housekeeping genes (2-ΔCt). Expression levels with standard deviations are reported in Figure 1 and Supplementary Table 1.

miR-9-3p and miR-19a-3p

miR-9-3p and miR-19a-3p were downregulated in PCa group with respect to BPH patients (P<0.001 and P<0.01, respectively) (Figure 1, Panel A). KLK3 and KLK11 were significantly up-regulated in PCa patients with respect to BPH (P<0.001 and P<0.01, respectively) (Figure 1, Panel A). KLK13 was not significantly different between PCa and BPH groups (Figure 1, Panel A). In the univariate logistic regression analysis, KLK11 reached the highest odds ratio (1.579, 95% CI 1.187-2.101) followed by KLK3 (odds ratio 1.575, 95% CI 1.229-2.020).

PCA3

PCA3 was not determined in 22 out of 38 BPH patients. In PCa group, PCA3 was detectable in all subjects, with mean expression value of -6.850 ± 3.405.

miR-9-3p and miR-19a-3p

miR-9-3p and miR-19a-3p were downregulated in PCa group with respect to BPH patients (P<0.001 and P<0.01, respectively) (Figure 1, Panel B). In the univariate logistic regression analysis, odds ratios were 0.719 and 0.820 for miR-9-3p and miR-19a-3p, respectively.

GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) was used to evaluate statistical significance between groups. Gene expression data were expressed as -ΔCt values. The significance threshold was set at 0.05. Mann-Whitney test was used to compare the expression levels of selected genes between groups. XLSTAT (Microsoft) was used to carry out Receiver Operating Characteristics (ROC) and logistic predictive models of selected markers in order to evaluate if their combination could ameliorate the proportion of correctly predicted diagnosis and to define cut-offs in the ΔCt values for a positive test. Logistic regression analysis was performed to estimate the accuracy of selected genes in predicting PCa. Prediction models were based on mRNA and miRNA expression values. First, univariate logistic regression analysis was made for each independent variable; differentially expressed genes between PCa and BPH groups were included. Bivariate logistic regression analysis was performed in order to establish the best combination of independent variables able to predict PCa.

miRNA target prediction and pathway analysis

Computationally predicted mRNA targets of the miRNAs under analysis were identified by TargetScan Release 6.2 (http://www.targetscan.org) [32] and MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm) [33]. The outputs of these target prediction algorithms were merged and all predicted targets (including overlapping targets) were revealed by Microsoft Visual FoxPro 9.0 (Microsoft Corp.) developer tool using the annotation database downloaded from the Ensembl homepage (www.ensembl.org) [34,35]. Targets of significantly differentially expressed miRNAs were subjected to pathway analysis in all pair-wise comparisons. DIANA mirPath software [36] analysis was also performed using DIANA-MicroT-4.0 and TargetScan 5 prediction algorithms.

Figure 1: Expression levels expressed as ΔCt (median with range and interquartile range) of selected miRNAs (KLK3, KLK11 and KLK13, Panel A) and miRNAs (miR-9-3p, miR-19a-3p, Panel B) between prostate cancer patients (PCa, left) and subjects with benign prostatic hyperplasia (BPH, right). ***P<0.001; **P<0.01; *P<0.05.

Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) was used to evaluate statistical significance between groups. Gene expression data were expressed as -ΔCt values. The significance threshold was set at 0.05. Mann-Whitney test was used to compare the expression levels of selected genes between groups. XLSTAT (Microsoft) was used to carry out Receiver Operating Characteristics (ROC) and logistic predictive models of selected markers in order to evaluate if their combination could ameliorate the proportion of correctly predicted diagnosis and to define cut-offs in the ΔCt values for a positive test. Logistic regression analysis was performed to estimate the accuracy of selected genes in predicting PCa. Prediction models were based on mRNA and miRNA expression values. First, univariate logistic regression analysis was made for each independent variable; differentially expressed genes between PCa and BPH groups were included. Bivariate logistic regression analysis was performed in order to establish the best combination of independent variables able to predict PCa.

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When treated individually, KLK3 showed the highest AUC (0.837), followed by miR-9-3p (0.769), KLK11 (0.739) and miR-19a-3p (0.723) (Table 3, Figure 3). The bivariate model which gave the highest specificity (76.92%) and sensitivity (86.67%) were obtained combining KLK11 with miR-19a-3p and miR-9-3p, respectively (Table 3).
miRNA target prediction and pathway analysis

 Computational target prediction of the 2 differentially expressed miRNAs has been performed with two algorithms: Target Scan Release 6.2 and Micro Cosm Targets Version 5. Among these predictions, 774 (for miR-9-3p) and 1044 (for miR-19a-3p) have been given by MicroCosm. 1237 (for miR-9-3p) and 1171 (for miR-19a-3p) transcripts with conserved sites were found in TargetScan. mRNA targets of differentially expressed miRNAs in all pair-wise comparisons have been subjected to pathway analysis to decipher the function of predicted miRNA targets in biological processes and to identify pathogenetic pathways potentially modified by posttranscriptional regulation. Results are shown in Figure 2.

Discussion

The early diagnosis of PCa is of crucial importance to allow for an effective treatment, which could improve cancer-specific survival [37]. Currently, diagnosis of PCa is performed through needle biopsy, whose indications are an elevated PSA level and/or a suspicious DRE [7]. The increase in PSA levels also occurs in benign conditions such as BPH, and this fact leads subjects with BPH and altered PSA levels to be subjected to useless and often repeated biopsies, with discomfort for the patient and high impact on sanitary costs. Both PCa and BPH are age-related conditions; this issue is particularly relevant considering the increasing age of population. The molecular mechanisms discriminating benign from malignant growth of prostate epithelial cells are partially unknown, although cellular overgrowth is a common event. The characterization of gene expression signatures exclusive for one of these two urological conditions could greatly improve the clinical management of PCa.

In the present report, the expression levels of 4 mRNAs (three kallikreins - KLK3, KLK11, KLK13 - and a PCa marker - PCA3) and 2 miRNAs (miR-9-3p, miR-19a-3p) were assayed in post DRE urine of men suffering PCa and compared with age-matched subjects with BPH. In these two categories of subjects, serum PSA levels were similar (p value 0.49), demonstrating that they do not represent a reliable discrimination tool. To our knowledge, this is the first report evaluating these molecules in post DRE urine sediments of PCa and BPH patients. We found a significant upregulation of KLK3 and KLK11 in cancer group, while KLK13 did not show significant differences. Higher levels of KLK3 and KLK11 mRNAs were positively correlated with risk of harboring PCa, as showed by univariate logistic regression analysis. A recent study on KLK3 mRNA levels in expressed prostatic secretions showed an increase in specimens obtained from PCa patients [38]. Contrary to these findings, the expression of KLK3 has been reported to be decreased in cancerous prostate tissue [39]. KLK11 and, to a lesser extent, KLK13, are both expressed at the mRNA and protein level in normal prostate tissue [40,41] and KLK11 mRNA is much present in PCa tissues with respect to BPH [42]. Higher KLK13 mRNA levels were demonstrated to be an independent favourable prognostic marker for breast carcinoma [43]. In contrast, another study reported the increase in expression of KLK13 as indicator of poor prognosis in epithelial ovarian carcinoma [44]. PCA3 gene, originally named DD3, was discovered in 1990s [45]. Its deregulation in PCa has been evaluated at the tissue level [45,46] and in post DRE urine sediments [46-48] also compared to subjects with BPH [49]. These studies also revealed that the use of this test might raise the specificity of diagnosis of PCa. In our study, PCA3 mRNA was detectable in all subjects of PCa group, whereas it was undetectable in 22 out of 38 patients with BPH. This issue made statistical comparisons between groups unfeasible.

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MicroRNAs represent another growing branching the research field of non-invasive diagnostic/prognostic cancer markers [26], because of their intrinsic stability in body fluids such as blood and urine [50]. The convergent alteration of the same sets of miRNAs in much pathology reflects their involvement in many crucial steps of carcinogenesis (Figure 2), miRNAs can act either as oncogenes or oncosuppressors, although their behaviour cannot be generalized to every type of cancer [51]. Several molecular events are potentially responsible for the alteration of miRNAs in cancer: among all, the imbalance of post-transcriptional regulation, genetic modifications...
and epigenetic alterations seem to play a major role. It is difficult to establish if a particular miRNA exerts its action as a tumor suppressor or oncogene, since this depends on many factors which vary according to tumor characteristic and other unknown contextual factors. We found both miR-9-3p and miR-19a-3p to be up-regulated in urine sediments from BPH group with respect to PCa patients. Many studies have demonstrated the epigenetic silencing of miR-9 loci in gastric tumors [52], breast cancer [53], non-small-cell lung cancer [54], oral squamous cell carcinoma [55] and renal cancer [56]. Contrary to these findings, miR-9 has been shown to increase in Hodgkin’s lymphoma [57] and c-myc-induced mammary tumors [58]; this is an interesting issue, since MYC is responsible for the balanced expression of many miRNAs, such as the oncogenic miR-17-19 cluster, also named oncomiR1 [26]. The miR-19 family belongs to the miR-17-92 cluster. The overexpression of miR-19a has been observed in prostate tumor tissues compared to adjacent benign tissue [59]. In prostate cancer cell lines, an upregulation has been shown in PC3 androgen-independent cell lines, whereas a downregulation was recorded in LNCaP androgen-dependent strain, compared to normal prostatic epithelial (PrEC) cells [60]. Recently, miR-19a was found to directly repress RAB13, which is able to tight cellular junctions, favouring metastasis formation in PCa [61]. These findings support our results since cancer patients included in the present study all harboured androgen-responsive and organ-confined tumors.

In our univariate logistic regression analysis, all differentially expressed genes between PCa and BPH groups showed acceptable confidence intervals (CI) of odds ratios. KLK3 and KLK11 mRNA levels positively correlated with risk of PCa (odds ratios > 1), whereas higher levels of miR-9-3p and miR-19a-3p trascripts were inversely correlated with cancer outcome (odds ratios < 1). The highest sensitivity and specificity was reached by miR-9-3p and miR-19a-3p, respectively. Bivariate logistic regression models were made in order to verify if a combination of variable could improve predictive power for PCa. The best model was obtained combining KLK11 with either miR-19a-3p or miR-9-3p, which significantly improved specificity (+14.06%) and sensitivity (+19.02%) of KLK11 alone. The highest AUC was reached combining KLK3 with miR-19a-3p. Our findings demonstrated that selected kallikreins and miRNAs proved to be an accurate diagnostic tool for PCa. Urine cells pellets obtained after DRE represent a reliable tool for PCa. Urine cells pellets obtained after DRE represent a reliable diagnostic marker for PCa. Urine cells pellets obtained after DRE represent a reliable diagnostic marker for PCa.

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