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Research Article

Differential expression of the *KLK2* and *KLK3* genes in peripheral blood and tissues of patients with prostate cancer

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Abstract

We used the multiplex semi-quantitative reverse-transcriptase PCR (RT-PCR) to investigate kallikrein 2 and 3 (*KLK2* and *KLK3*) mRNA levels in prostate tissue from 42 prostate cancer patients, 33 of whom were also assessed for peripheral blood *KLK2* expression by qualitative semi-nested RT-PCR. We found that *KLK2* was an important tissue biomarker for distinguishing between prostate cancer patients and those with benign prostatic hyperplasia, particularly when *KLK2* expression was > 60% of that of the β2-microglobulin constitutive gene. Patients with an average relative expression value ≥ 0.6 (cutoff value) had an eleven-fold higher chance of having prostate cancer. When one or two tissues samples were evaluated for *KLK2* expression using the cutoff value the estimated chance of having prostate cancer was increased by seven times for one positive sample and 45 times for two positive samples. There was no significant correlation between *KLK3* gene expression and prostate cancer diagnosis. Logistic regression for blood and tissue *KLK2* expression successfully detected 92% of the prostate cancer cases. The detection of *KLK2* in blood showed a sensitivity of 59% and a specificity of 82%. This study indicates that the *KLK2* gene may be a useful molecular marker for the diagnosis of prostate cancer and that analysis of *KLK2* expression in blood and tissues could provide a novel approach for the clinical investigation of this type of cancer.

Key words: kallikrein II, molecular markers, prostate cancer, PSA, semi-quantitative RT-PCR.

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Introduction

Prostate cancer (PCa) has become one of the most common diseases among elderly men. Prostate-specific antigen (PSA) is a glycoprotein found in normal, hyperplasic and tumoral prostate tissues, seminal liquid and the blood of patients with prostate cancer (Wang *et al.*, 1979). Although PSA is not exclusive to the prostate (Diamandis and Yu, 1997), extra-prostatic protein has little or no interference on the clinical analysis of prostate cancer (Rittennhouse *et al.*, 1998).

As with PSA, high levels of the prostatic kallikrein hK2 have also been found only in the prostate (Black *et al*., 2000) and Lyon *et al.* (1995) have associated hK2 activity with prostate cancer invasion and metastasis. In addition, hK2 shows physiological synergism in the regulation of PSA activation through its ability to convert the pre-PSA form into mature PSA (Takayama *et al.*, 1997).

The search for molecular markers that could be used for the early diagnosis of prostate cancer has become one of the most important objectives in clinical investigation, particularly because the current methods are invasive, show low specificity and require additional procedures for therapeutic decisions. During the work described in this paper we examined the expression of the *KLK2* and *KLK3* genes in prostate tissue and the expression of the *KLK2* gene in the peripheral blood of patients diagnosed with prostate cancer and benign prostatic hyperplasia (BPH) and assessed the potential use of these genes as biomarkers in the clinical diagnosis of prostate cancer.

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Patients and Methods

Patients, sampling and clinical classification

This study was carried out in 2002 to 2003 in the Molecular Genetics Laboratory of the Federal University of Uberlândia (UFU), in collaboration with the Urology Service of the University Hospital and was approved by the UFU Ethics Research Committee (protocol number 005/2001) which included informed consent by the patients. Forty-two patients (median age 69, range 49 to 87 years) were assessed by two pathologists and histologically classified as follows: 14 with BPH, 15 with organ-confined prostate cancer (pT1a to pT2c), and 13 with extra-capsular invasion prostate cancer (pT3a to pT4b) that will be considered for analysis purposes as a metastatic group. Patients presenting prostate intraepithelial neoplasia (PIN) and/or prostatitis were excluded from the investigation. Most BPH patients were submitted to transurethral resection of the prostate (TURP), the exception being 2 patients that underwent open prostatectomy. All prostate cancer patients were submitted to radical prostatectomy after being selected based on the following criteria: Gleason score for the biopsy of less than 8, negative X-rays and bone scan analyses and a rectal examination compatible with organ confined cancer. Two samples of fresh prostate tissue from different regions of the organ of each patient were carefully selected using histological examination to investigate *KLK2* and *KLK3* genes expression levels. In 11 BPH and 22 prostate cancer patients, a peripheral blood sample was collected before surgery. Sera total PSA (tPSA) concentrations were detected using the IMMULITE® chemiluminescent immunoassay system (Diagnostic Product Corporation, Los Angeles, USA).

Total RNA extraction and reverse transcription

Total RNA was extracted from peripheral blood leukocytes and macerated prostate tissue using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Inc.). Reverse transcription (RT) was accomplished by adding 1 µg of total RNA from the individual blood or tissue samples to a final volume of 20 µL (completed with diethylpyrocarbonate (DEPC) treated water) containing 10 units of RNase inhibitor, 40 units of MMLV reverse transcriptase (RT), 1x MMLV-RT buffer, 200 µM of each dNTP and 6 µM of random hexamer primers and the solution incubated at 37 °C for 1 h and then 95 °C for 5 min.

Multiplex semi-quantitative RT-PCR of the prostate tissue samples

The cDNA was PCR co-amplified using two different primer pairs for the target genes, according to their GenBank accession sequence numbers. For the *KLK2* gene (accession number AF1188747) the primers were: sense 5'-CAGCATCGAACCAGAGGAGT-3', nucleotide position 490-509, and antisense 5'-ACTAGAGGTAGGGG TGGGAC-3', nucleotide position 810-829. For the *KLK3* gene (accession number X05332) the primers were: sense 5'-TCCAATGACGTGTGTGCGCA-3', nucleotide position 581-600, and antisense 5'-CCTTGATCCACTTCCG GTAA-3', nucleotide position 787-806. The constitutive *beta2-microglobulin* gene (β-*2M*): 5'-AGCAGAGAATG GAAAGTCAAA-3' and 5'-TGTTGATGTTGGATAAG AGAA-3') was used as an internal positive control to normalize the products of the amplification reactions. To check for genomic DNA contamination PCR reactions were also performed using total RNA as template, but no amplification was observed demonstrating that the samples had no contaminant genomic DNA. Additionally, primers were designed for selective amplification of RNA, in which both primer ends (5' and 3') belonged to two adjacent exons.

Amplification was carried out by adding 2 µL of primary cDNA to a 25 µL PCR mixture consisting of 200 µM of each dNTP, 0.4 µM of the primer pair for *KLK2* or *KLK3*, 0.96 µM of the primer pair for β-*2M*, 2.0 mM $MgCl_2$, 1.5 unit of Taq DNA polymerase and 1x buffer. The reactions were incubated at 95 °C for 3 min, followed by 29 cycles at 95 °C for 30 s, 59 °C for 40 s and 72 °C for 40 s, with a final extension of 10 min at 72 °C. The ideal number of PCR cycles (29) was determined when the co-amplification of both genes reached the exponential phase.

Semi-nested RT-PCR of peripheral blood

For these amplifications 2 µL of cDNA was added to a 25 µL PCR mixture containing 200 µM of each dNTP, 0.4 mM of the same primer pair used to amplify *KLK2* in the tissue samples, 1.5 mM $MgCl_2$, 1.5 units of Taq DNA polymerase and 1x buffer. The reaction conditions consisted of 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 66 °C for 40 s and 72 °C for 40 s, with a final extension of 10 min at 72 °C. All of the samples were co-amplified with the β-*2M* gene as described above.

In the next step, 2 µL the first PCR amplification was added to a 25 µL mixture containing 200 µM of each dNTP, 0.4 mM of the semi-nested pair of primers for the *KLK2* gene (accession number AF1188747: sense 5'-AGTTCTT GCGCCCCAGGAGT-3', nucleotide position 507-526, and antisense 5'-ACTAGAGGTAGGGGTGGGAC-3', nucleotide position 810-829), 1.5 mM $MgCl_2$, 1.5 units of Taq DNA polymerase and 1x buffer. Re-amplification reactions were accomplished under the following conditions: 95 °C for 3 min, followed by 20 cycles at 95 °C for 30 s, 62 °C for 40 s and 72 °C for 40 s, with a final extension of 10 min at 72 °C. The PCR reactions were standardized for 20 cycles so that circulating *KLK2* mRNA in BPH patients could not be detected.

Relative levels of gene expression as assessed by densitometry

The *KLK2*, *KLK3* and β-*2M* gene amplicons obtained were analyzed and quantified based on the staining intensities of the corresponding bands as assessed using the ImageMaster VDS software program, version 2.0 (Amersham Biosciences). The relative levels of *KLK2* and *KLK3* were obtained for each sample by normalizing the densitometric readings using the ratio target mRNA/β-*2M* mRNA, where target mRNA represents the *KLK2* or *KLK3* values.

Statistical analysis

All the statistical analyses were performed using the Statistica 99 Edition software (Anon, 1999). Normality of the data was verified using the Shapiro-Wilk test. Logistic regression was applied to the relative mRNA levels of the blood and tissue sample in order to determine whether the target genes were able to detect prostate cancer and also to distinguish between organ confined and metastatic disease. A logistic regression of the combined blood and tissue data from the same patient was used to estimate the diagnostic value of these markers. The Kruskal-Wallis test was used to compare the relative levels of expression of both genes between samples at a probability level of 5%. Pearson's correlations were applied to all clinical laboratory parameters. Odds ratios were also calculated for each marker to determine the chance of having prostate cancer. The accuracy (A), sensitivity (S), specificity (E), positive predictive value (PPV) and negative predictive value (NPV) of both molecular markers in diagnosing prostate cancer and BPH were compared with the pathological findings.

Results

The semi-quantitative analyses performed for both markers (Figure 1) in the two prostate tissues of some patients have shown different mRNA expression levels, which may indicate the heterogeneous nature of the prostate cancer, and suggesting that tissues are under different disease stages.

There was a significant correlation ($r = 0.476$; $p < 0.04$) between tissue and peripheral blood *KLK2* expression and the clinical parameters tPSA, Gleason score, TNM (Tumor, lymph Node and Metastasis) classification and age, but the *KLK3* and β-*2M* markers showed no correlation. The alternative splicing form of the *KLK2* gene (present as a very low intensity 378-bp band) was observed in all patients but with no differences between samples, and was therefore not included in the analysis. A significant correlation ($r = 0.55$; $p < 0.01$) was also observed between TNM classification and Gleason score (Table 1).

The relative expression levels of *KLK2* and *KLK3* (Table 1) were not normally distributed ($p < 0.01$) and logistic regression analysis of the relative levels of *KLK2* and *KLK3* in prostate tissue showed that only *KLK2* was a good marker for predicting prostate cancer (*KLK2*, $p = 0.00001$; *KLK3*, $p = 0.298$). Furthermore, *KLK2* expression in prostate tissue was associated with the disease staging while *KLK3* expression was highly variable within stages (*KLK2*, $p = 0.00053$; *KLK3*, $p = 0.47$) (Figure 2).

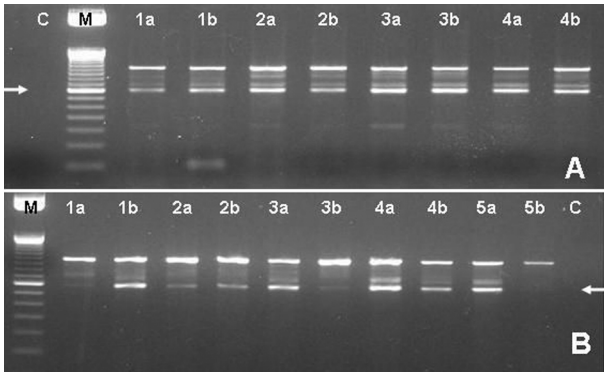

Figure 1 - Semi-quantitative multiplex RT-PCR for *KLK2* gene expression analysis of two samples of prostate tissues from patients with (A) prostate cancer (PCa) or (B) benign prostatic hyperplasia (BPH). We show the results for four PCa and five BPH patients each represented by two samples (lanes a and b) produced from tissue from independent prostate regions. The samples were analyzed in a multiplex reaction with a 534-bp positive fragment of the β-*2M* constitutive gene. C = negative control reaction for DNA contamination. M = 50-bp ladder molecular marker. The white arrow indicates the 341-bp *KLK2* gene fragment. The faint band (378 bp) over the 341-bp fragment is the alternative splicing of the *KLK2* gene.

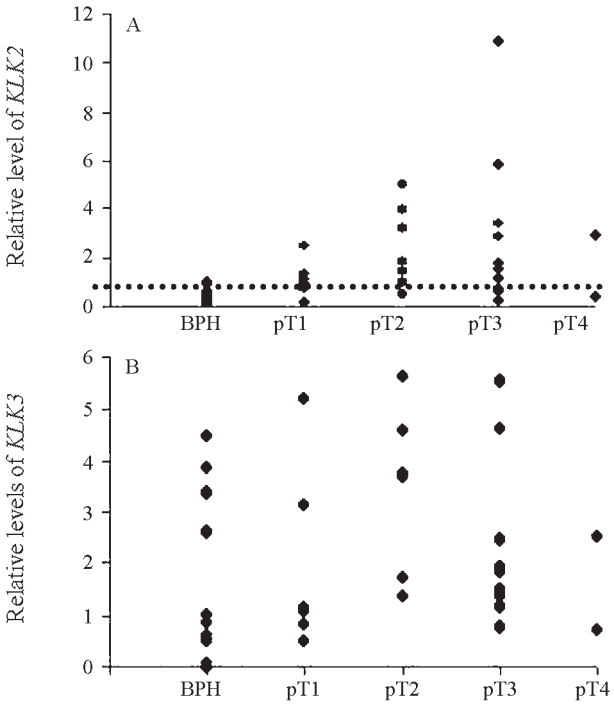

Figure 2 - Relative expression of *KLK2* (A) and *KLK3* (B) genes in patients according to their TNM (Tumor, lymph Node and Metastasis) tumor staging (pT1, pT2, pT3 and pT4) and benign prostatic hyperplasia (BPH) classification. The dashed line represents the 0.6 *KLK2* cutoff expression limit for discriminating prostate cancer patients from BPH patients.

Table 1 - Clinical parameters and laboratory data for expression analyses of the *KLK2* and *KLK3* genes in prostatic tissues and peripheral blood.

Patient	*KLK2* expression in prostatic tissue[1]	*KLK3* expression in prostatic tissue[1]	Total Serum PSA (ng mL^{-1})[2]	*KLK2* expression in peripheral blood[3]	Clinical staging (TNM score)[4]	Gleason score[5]	Age
1	0.9	4.48	12.0	No	BPH	-	49
2	0.0	0.85	4.0	No	BPH	-	58
3	0.24	0.55	3.0	No	BPH	-	79
4	0.46	0.85	2.5	No	BPH	-	66
5	0.62	2.62	10.7	No	BPH	-	61
6	0.0	0.0	1.1	No	BPH	-	73
7	0.1	0.59	18.5	No	BPH	-	81
8	0.97	3.35	2.5	Yes	BPH	-	87
9	0.0	0.02	2.2	No	BPH	-	66
10	0.14	4.48	2.1	Yes	BPH	-	68
11	0.08	2.68	5.4	No	BPH	-	60
12	0.45	0.59	10.0	-	BPH	-	74
13	0.95	3.87	3.10	-	BPH	-	76
14	0.41	0.99	3.5	-	BPH	-	80
15	1.45	3.47	8.04	No	pT1c pNo pMo	7(3+4)	67
16	2.49	1.07	5.9	Yes	pT1a pNo pMo	6(3+3)	74
17	0.97	4.57	9.6	Yes	pT2c pNo pMo	7(4+3)	71
18	0.4	0.85	5.9	No	pT2a pNo pMo	-	70
19	2.5	0.83	6.5	Yes	pT1a pNo pMo	5(3+2)	71
20	0.19	1.13	8.8	No	pT1a pNo pMo	-	66
21	0.94	5.2	11.1	Yes	pT2c pNo pMo	6(3+3)	66
22	1.32	1.53	5.4	No	pT1a pNx pMx	5(2+3)	59
23	0.5	3.68	5.3	No	pT1c pNo pMo	7(3+4)	66
24	0.71	0.5	8.71	No	pT2a pNo pMo	7(3+4)	66
25	3.19	3.74	22.2	Yes	pT1c pNo pMo	6(3+3)	75
26	3.98	5.65	8.59	Yes	pT2c pNo pMo	6(3+3)	78
27	1.08	3.14	1.2	Yes	pT1a pNo pMo	4(2+2)	47
28	1.83	1.7	5.5	-	pT2c pNo pMo	5(3+2)	70
29	5.02	1.36	6.0	-	pT2b pNo pMo	6(3+3)	68
30	0.2	5.54	5.5	No	pT3a pNo pMo	6(3+3)	63
31	5.81	1.36	5.7	Yes	pT3c pNo pMo	7(4+3)	80
32	1.76	1.55	7.5	Yes	pT3c pNo pMo	7(4+3)	62
33	0.38	0.69	7.1	Yes	pT4a pNo pMo	7(4+3)	57
34	2.89	2.49	192	Yes	pT3c pNx pMx	-	74
35	3.42	1.15	9.18	Yes	pT3b pNo pMo	9(5+4)	75
36	0.63	0.77	18.0	No	pT3c pNo pMo	9(5+4)	69
37	1.13	1.9	16.5	Yes	pT3a pNo pMo	7(3+4)	61
38	1.56	4.61	8.5	No	pT3a pNo pMo	6(3+3)	46
39	10.9	2.45	7.6	-	pT3a pNo pMx	7(4+3)	63
40	2.88	1.84	13.0	-	pT3b pNo pMo	7(4+3)	67
41	0.66	2.46	47.0	-	pT3b pNo pMo	7(4+3)	76
42	0.7	1.49	8.2	-	pT3c pN1 pMo	7(4+3)	61

[1]Semi-quantitative RT-PCR values of *KLK2* and *KLK3* gene expression levels in prostatic tissues are given by the ratio between the expression levels of target genes and the β2-microglobulin gene. [2]PSA = Prostate-specific antigen. [3]Qualitative semi-nested RT-PCR analysis of *KLK2* gene expression in peripheral blood. A dash (-) indicates 'data not available'. [4]TNM = Tumor, lymph Nodes, Metastasis classification system. The cancer stages advance from pT1 (early stage) to pT4 (late stage); BPH= benign prostatic hyperplasia. [5]The Gleason score is a scale of 2 to 10 (worst prognosis) and is composed of the sum of two Gleason grades which assesses prostate tumor architecture on a scale from very well differentiated (grade 1) to very poorly differentiated (grade 5). For example, patient 35 had a Gleason score of 9 composed of two Gleason grades (5+4), 5 being the most prominent architecture and 4 the second most prominent architecture. A dash (-) indicates 'data not available' or, in the case of BPH, inapplicable.

As explained above, the relative levels of *KLK2* and *KLK3* expression were obtained for each sample by normalizing the densitometric readings using the ratio target mRNA/β-*2M* mRNA, where target mRNA represents the *KLK2* or *KLK3* values. The relative *KLK2* expression levels were significantly higher in prostate cancer tissues than in BPH ($p = 0.0001$). A cutoff value of 0.6, representing 60% of the *KLK2* expression in relation to the β-*2M* gene, was calculated by logistic regression and maximized the clinical classification of patients as having prostate cancer or BPH. Individuals with an average *KLK2* gene expression level ≥ 0.6 (considered positive for prostate cancer) had eleven times (95% CI_{KLK2} = 2.5 to 52.0) higher chance of having a tumor. The estimated chance of occurrence was seven times higher (95% CI_{KLK2} = 1.3 to 43.0) when one tissue sample was independently positive, and was 45 times higher (95% CI_{KLK2} = 4.0 to 500) when two tissue samples were independently positive for *KLK2* gene expression.

Of the 42 patients whose prostate tissues were screened by semi-quantitative RT-PCR, 27 were positive (four BPH, 12 organ-confined prostate cancer and 11 metastatic prostate cancer) based on a *KLK2* cutoff value of 0.6. The Table 2 is showing the clinical performance parameters (accuracy, sensitivity, specificity, positive predictive value, and negative predictive value) as calculated based on the detection limit of gene transcripts (cutoff value) in tissue samples.

Peripheral blood analyses showed that the *KLK2* and tPSA markers were reliable indicators (*KLK2*, $p = 0.02$; tPSA, $p = 0.01$) of prostate cancer as compared to BPH (Figure 3).

Since the peripheral blood levels of *KLK2* mRNA were higher in patients with prostate cancer than those with BPH, we standardized the semi-nested RT-PCR cycles to detect *KLK2* expression in the circulating blood cells of most prostate cancer patients. Of 33 blood samples analyzed, 15 gave a positive reaction (two BPH, 7 organ-confined prostate cancer and 6 metastatic prostate cancer). Based on the TNM disease stage, the frequency of false negatives was 50% for the pT1 stage (four patients), 40% for pT2 (two patients), 37.5% for pT3 (three patients) and 0% for pT4.

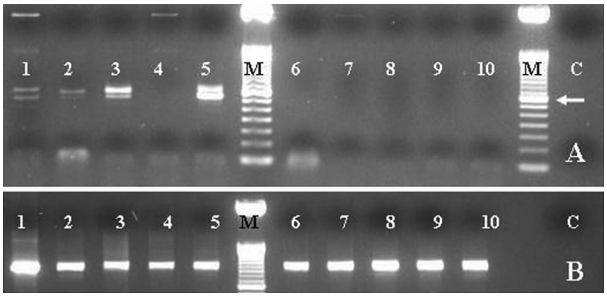

Figure 3 - Expression of *KLK2* and β-*2 microglobulin (*β-*2M)* mRNA in peripheral blood of prostate cancer and benign prostatic hyperplasia (BPH) patients. A: semi-nested RT-PCR for *KLK2* gene expression. Columns 1 to 5 represent prostate cancer patients, and columns 6 to 10 are BPH patients. M = 50-bp ladder (molecular marker) and the arrow a 350-bp marker. C = negative control reaction (without template). The *KLK2* fragments sizes were 324 and 361bp. B: RT-PCR reaction for the β-*2M* gene as a positive control for each sample, generating a 534 bp fragment.

The detection of *KLK2* mRNA in the circulation was associated with a 6.5-fold greater chance of having prostate cancer (95% CI_{KLK2} 1.12 to 37.48). The qualitative results of *KLK2* detection in peripheral blood were also analyzed to determine its clinical performance (Table 2).

Logistic regression of the combined results for *KLK2* expression in blood and tissue samples from prostate cancer and BPH patients successfully identified 92% of all prostate cancer cases, with 59% being true positive blood samples and 33% true positive biopsy samples. Of the 41% false negative blood samples, 79% were correctly identified using the same biomarker in prostate tissue (PPV = 87%).

Discussion

To determine the potential usefulness of *KLK2* and *KLK3* as biomarkers in the diagnosis of prostate cancer we used multiplex semi-quantitative RT-PCR to detect mRNA in prostate tissues and semi-nested RT-PCR to detect mRNA in peripheral blood cells. The use of hexamer primers allowed normalization of the RNA amplification products and ensured that there were corrections for variation between reactions.

It is important to emphasize that quantitative analysis of mRNA can be achieved by several RT-PCR approaches, which can be divided into comparative and absolute quantitative PCR (Q-PCR), both of which can use either competitive PCR or real time PCR with fluorescent probes/primers. Competition assays can be used to compare expression levels of the same gene in different samples, while the absolute Q-PCR technologies can use standard curves to estimate the specific amount of a specific target (Rose' Meyer *et al.*, 2003). Determination of cycle threshold (CT) in real time thermocyclers uses a similar approach to that used in semi-quantitative analysis in conventional multiplex PCR, for which values are calculated based on an endogenous standard which is usually a housekeeping gene such as the

Table 2 - Calculation of clinical performance parameters of total serum prostate-specific antigen (tPSA) and the molecular markers *KLK2* and *KLK3* in peripheral blood and prostate tissues for prostate cancer diagnostics.

Clinical performance parameters	*KLK2* tissue	*KLK2* blood	*KLK3* tissue
Accuracy	79%	67%	74%
Sensitivity	82%	59%	82%
Specificity	71%	82%	57%
Positive predictive value	85%	87%	79%
Negative predictive value	67%	50%	62%

β-*2M* gene. The use of this endogenous standard in the RT-PCR assay provides a direct comparison between multiple samples and has several other advantages. Firstly, its detection after RT and PCR indicates the success of these two steps. Secondly, the amount of cDNA corresponding to the endogenous marker is an indicator of the degree of degradation and purity of the sample. Thirdly, the internal control compensates for the inherent inter-assay variability of RT-PCR. In fact due to the exponential nature of PCR, a small variation in amplification efficiency dramatically affects the yield of amplification product (Pernas-Alonso *et al.*, 1999).

It has been shown that results of absolute Q-PCR data analysis have validated the results of comparative data analysis that used an internal control, which means, the copy number of mRNA molecules correlated significantly with comparative data. Therefore, a comparative analysis is an adequate and consistent procedure to investigate gene expression levels and is not dependent upon absolute levels of expression (Rose' Meyer *et al.*, 2003).

In selecting a technique or biomarker to analyze a molecular event, it is essential to know of the existence of post-transcriptional or post-translational alterations (Favre *et al.*, 1997). An understanding of these events is an important step in the selection of potential biomarkers for the early diagnosis of prostate cancer and for disease staging by looking for differential gene expression during tumor development.

We found that *KLK2* mRNA expression was greater in prostate cancer tissue compared to BPH tissue, whereas there were no differences in *KLK3* expression. Indeed, the detection of *KLK3* gene expression in prostatic tissue is a controversial issue, with the levels being greater in benign than in malignant tissue (Magklara *et al.*, 2000; Herrala *et al.*, 2001). However, low *KLK3* expression in tumoral tissues may be associated with the development of more aggressive tumors (Stege *et al.*, 2000), while others have found no differences in *KLK3* gene expression in prostate cancer and BPH tissues (Henttu *et al.*, 1990). Larger concentrations of PSA have been observed in tumor tissues compared to BPH tissues, based on immunohistochemical analyzes using monoclonal and polyclonal antibodies (Darson *et al.*, 1999).

The expression of *KLK2* is also a matter of controversy, with some authors (Herrala *et al.*, 1998; Herrala *et al.*, 2001) having conducted immunohistochemical studies which detected overexpression of the *KLK2* gene in prostate cancer, while, in contrast, Magklara *et al.* (2000) reported *KLK2* expression to be higher in BPH as compared to prostate cancer, whereas Henttu *et al.* (1990) found no differences in *KLK2* expression between BPH and prostate cancer. Such discrepancies between results are most likely to be due to a variety of factors, including different antibodies being used in different studies, variation in the technologies and equipment used and the operators, and the heterogeneous nature of the cancer and tissues.

We found that the 0.6 cutoff for *KLK2* tissue expression efficiently distinguished prostate cancer from BPH, with the chance of a reliable clinical diagnosis being greater as the amount of tissue used increased.

The tissue samples used by us were obtained during radical prostatectomy and not from biopsies, hence it is possible that gene expression was underestimated since the tissues were not obtained by microdissection. However, this does not invalidate the clinical use of *KLK2* as a marker since tumors are generally heterogeneous and multifocal, and microdissection would not provide a very representative histological analysis.

As shown in this paper, peripheral blood can be used instead of tissue samples in preliminary analysis. Since the *KLK2* and *KLK3* genes occur almost exclusively in prostatic epithelial cells (Rittennhouse *et al.*, 1998), any release of these cells into the circulation as a result of glandular rearrangements can be detected by the sensitive methods described here. The greater the tissue trauma, the greater the number of prostatic cells released into the blood.

However, false negatives can also be observed, and a possible explanation for this result in peripheral blood could be that, in the early stages of the tumor, malignant cells may be masked by the greater number of normal cells released into the circulation, thereby apparently diminishing the levels of *KLK2* expression. On the other hand, in more advanced tumors, some cell lines may be transformed from being hormonal dependent to being hormonal independent, thus inhibiting *KLK2* and *KLK3* expression. This could explain the overexpression of these genes in prostate androgen-dependent tumor cells compared to androgen-independent cells (Black *et al.*, 2000; Vaarala *et al.*, 2000).

Serum total PSA values of < 4 ng mL^{-1} do not guarantee the absence of tumors since 22% of patients with organ-confined disease have tPSA values below this level (Catalona *et al.*, 1997). In addition, the 4 ng mL^{-1} cutoff limit for tPSA used as an indicator for a prostate biopsy procedure has been shown in the literature to have a positive predictive value of 31 to 54% (Brawer and Kirby, 1998). In our data, the *KLK2* biomarker in blood had a positive predictive value of 87%, suggesting that our proposed procedure would prevent unnecessary biopsies by reducing from 46% to 13%.

The lack of agreement between the pathological findings and *KLK2* detection may reflect the fact that prostate cancer is multifocal and multifactorial, and that different *KLK2* expression patterns may exist in different phenotypes and clinical stages of the disease.

Our semi-quantitative and qualitative analyzes of *KLK2* showed that this gene was a good biomarker for distinguishing prostate cancer from BPH and could be used with both prostatic tissue and peripheral blood cells.

We propose the use of the *KLK2* biomarker in blood, together with the quantification of serum tPSA levels, in order to improve the investigation of the clinical status of patients. Further studies should be carried on to demonstrate its true value as marker for disease staging.

Acknowledgments

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