Simultaneous Quantification of Human Glandular Kallikrein 2 and Prostate-Specific Antigen mRNAs in Peripheral Blood from Prostate Cancer Patients

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We present a multiplexed and internally calibrated quantitative reverse transcription-PCR (QRT-PCR) assay to detect human glandular kallikrein 2 (hK2) and prostate-specific antigen (PSA) transcripts in blood samples from healthy subjects and prostate cancer (PC) patients. The assay detected 50 copies of hK2 and PSA mRNA, and 1 PSA- and 10 hK2-expressing LNCaP cells in the presence of 2.5×10^6 PSA- and hK2-negative cells. In PC patients, 20 of 25 and 19 of 25 gave detectable PSA and hK2 mRNAs, respectively. Number of hK2 mRNA copies was significantly higher than that of PSA mRNA copies in patients with biochemically progressive ($P = 0.02$) PC, and with locally advanced and metastasized ($P = 0.004$) PC. Patients with rapidly progressive and hormone refractory PC gave detectable hK2 mRNA only in 2 of 8 and PSA mRNA in 3 of 8 patients. Neither PSA nor hK2 mRNAs were detected in 16 healthy subjects. PSA and hK2 discriminated PC patients with biochemically progressive and advanced disease from the controls and from the aggressive distant metastatic disease. The assay provides a reliable quantification of the number of hK2 and PSA mRNA copies, allows to discriminate PC cases from healthy subjects, and offers a tool for further studies on molecular staging of PC. (*J Mol Diag* 2001, 3:111–122)

Prostate cancer (PC) is one of the most common malignancies in men in western countries. Recently, the incidence of PC has increased due to enhanced diagnostic tools and efficient screening programs that are usually based on digital rectal examination and serum prostate-specific antigen (PSA) test. The serum PSA screening is capable of detecting organ-confined or localized cancers that may be cured surgically.¹ Detection of advanced cancers is also important because metastatic malignancies require different therapeutic approaches than the organ-confined cancers.² However, the number of “micrometastatic” circulating tumor cells can be very

small and therefore the minimal spread of the cancer is difficult to detect by routine examinations. Traditionally, metastatic cancer cells have been detected in lymph node samples using histological and immuno-histochemical techniques,³ but this has led to a need for new methods capable of detecting even smaller number of circulating cancer cells to identify more accurately patients with extraprostatic disease.

During the last few years, new reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed aiming to identify extraprostatic tumor cells by detecting mRNA markers such as PSA,^{4–9} human glandular kallikrein 2 (hK2),^{10–12} and prostate-specific membrane antigen (PSMA)^{13,14} to mention the most commonly used markers. The assays have been validated with different biological samples, such as peripheral blood, lymph nodes, and bone marrow from PC patients. Most of the assays have been designed to amplify the reverse transcribed target with one round of PCR or with nested-PCR consisting of two rounds of PCR and several tens of amplification cycles. Thereafter, the amplification products are detected by gel electrophoresis and radioactive labeling. These assays provide qualitative results (positive or negative result in respect to the PCR product) and commonly use the expression of a housekeeping gene to evaluate whether the quality of RNA is good enough for the RT-PCR amplification. Hence the results from different RT-PCR studies aiming to prove the clinical usefulness of the method have been controversial.^{15–21} Many of the research groups have stated that the large variations in the results may be due to the various RT-PCR assays each designed and validated differently in the absence of the basis of international standardization of the procedures, thus proposing the need for more standardized and quantitative RT-PCR (QRT-PCR) assays.^{22–24}

The first QRT-PCR assays exploited endogenous RNA standards expressed in the cell.²⁵ Usually the endoge-

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nous standard is mRNA expressed by a housekeeping gene, such as β -actin or glyceraldehyde 3-phosphate dehydrogenase. The endogenous standard can be co-amplified with the target mRNA using their own primers in the same RT-PCR reaction, thus controlling the amplification steps. The amount of target mRNA is expressed as a ratio of target to standard signals, because the number of endogenous mRNA standard copies are not known; therefore, this approach is often called as semiquantitative. In addition to the fact that the exact number of target mRNA molecules cannot be shown, another drawback is that it will not be reproducible and accurate to determine the rare or low copy number target mRNA using the common or high copy number endogenous standard mRNA.

Lately, the commonly used standard has been an exogenous RNA or DNA containing sequences of the target mRNA hence allowing co-amplification of the standard and the target with the same primers and the same amplification efficiency of the two. The first assays with the exogenous standard used an approach in which serial dilutions of standard with a known amount of molecules were mixed with a constant amount of sample.^{26–28} This approach requires multiple tubes for the analysis of one sample, and the quantification of the target is based on the determination of an equivalency point between the amount of the exogenous standard and the target amplification products. At present, some of the developed assays exploit an external calibration curve and need only one tube for the analysis of one sample.^{29–31} In this case, a constant amount of exogenous standard is mixed with the samples and the calibrators, and the target transcripts are quantified after amplification by calculating the target to standard ratio in the sample and comparing the ratio to that in a calibration curve. In addition, if the exogenous mRNA standard is used, it will be possible to control the variations of an assay starting from the RNA extraction step.⁹

We have recently presented a QRT-PCR assay for the detection of PSA mRNA^{9,24} and our present aim was to develop a QRT-PCR assay for the simultaneous detection of hK2 and PSA mRNAs in blood samples. The QRT-PCR assay uses an external calibration curve and two highly target-like exogenous, internal standard (IS) mRNAs, IS-hK2 and IS-PSA for the specific quantification of hK2 and PSA mRNAs, respectively. To study the detection of hK2 and PSA in biological samples the multiplexed QRT-PCR assay was applied on cultured LNCaP cell samples and blood samples from 16 healthy volunteers and 25 PC patients.

Materials and Methods

Cell Lines

The PSA- and hK2-expressing human prostatic carcinoma cell line LNCaP and the non-PSA- and non-hK2-producing mouse myeloma cell line SP2/0 were obtained from the American Type Culture Collection. The cell lines were cultured in flasks containing Dulbecco's modified Eagle's medium (Life Technologies, GIBCO BRL, Grand

Island, NY) with 100 ml/L fetal bovine serum (Hyclone, Logan, UT), and maintained in a 5% CO₂ incubator at 37°C. The medium for LNCaP culture was supplemented with 1 nmol/L synthetic hormone methyltrienolone (R1881) (New England Nuclear, Boston, MA). The cells were grown until near confluency, detached, washed with phosphate-buffered saline, and counted. Unlike the SP2/0 cells, the LNCaP cells were detached by trypsin-EDTA treatment. The SP2/0 samples of 2.5×10^6 cells and the dilutions of LNCaP cells were stored at -70°C until RNA extraction.

Blood Specimens

Blood samples (EDTA) of 5 ml were collected from 25 patients with PC, 9 healthy women, and 7 healthy men volunteers. The healthy volunteers were included as controls in the study. The patients were divided into three groups based on their disease stage. Group A ($n = 4$) consisted of patients with biochemically progressive PC as detected by rising serum PSA. Patients in group A were under watchful waiting and no metastases had been detected. Group B ($n = 13$) consisted of patients with locally advanced and metastasized (regional lymph node and bone metastases) disease under hormonal treatment. Group C consisted of hormonally treated patients ($n = 8$) with rapidly progressive, hormone refractory PC and multiple distant metastases. The study protocol was in accordance with Helsinki Declaration of 1975, as revised in 1983. Nucleated blood cells were isolated as described earlier,⁹ and after the isolation the cell pellets were snap-frozen in liquid nitrogen and stored at -70°C until RNA extraction. Serum total PSA values were available for all prostate cancer patients except for one patient in group B and for two patients in group C.

Oligonucleotides

The synthesis of PCR primers, detection probes and DNA targets, and the biotinylation of the 3' PCR primer and the DNA targets were carried out as reported previously (Table 1).^{9,24} The detection probes for hybridization assay contained additional diaminohexanedeoxycytidines to be labeled with lanthanide chelate as described earlier.^{32–34} The detection probes for hK2 and PSA were labeled with the Eu³⁺ chelate, and the probes for the IS-hK2 and IS-PSA were labeled with the Tb³⁺ chelate.

Construction of pGEM3-hK2 and pGEM3-IS-hK2 cDNA Plasmids

Full-length hK2 cDNA was amplified by PCR (primers A and B, Table 1) from human prostate cDNA library with λ gt11 clones encoding hK2.³⁵ The 5' (A) and 3' (B) primers were designed to contain *Eco*RI and *Kpn*I restriction sites in the 5' end of their sequence, respectively. A 100- μ l PCR reaction contained 20 mmol/L Tris-HCl, pH 8.8; 10 mmol/L KCl; 10 mmol/L (NH₄)₂SO₄; 2 mmol/L MgSO₄; 1 ml/L Triton X-100; 1 mg/ml nuclease-free BSA;

Table 1. Oligonucleotides Used in This Study

Oligonucleotide	Name	Sequence from 5' to 3' end	Nucleotide position*	Label	Labels/ oligo- nucleotide
Construction of pGEM3-hK2					
5' primer	A	ATATGAATTCATGTGGGACCTGGT [†]	43–56	— [†]	—
3' primer	B	CTATGGTACCTTTTTTTTTTTTTTTTAAACCAC [‡]	1497–1523	—	—
Construction of IS-hK2					
5' primer [§]	A	ATATGAATTCATGTGGGACCTGGT			
3' primer	C	GATGGTGTCTTGATCCACTT	793–813	—	—
5' mutation primer	D	TGTGCTAGATTACTCTGAGAAGGTGA	592–600 603–619	—	—
3' mutation primer	E	TTCTCAGAGTAATCTAGCACACATGTC	546–600 603–614	—	—
PCR					
hK2 and IS-hK2 5' primer	F	GAACCAGAGGAGTTCTTGCG	523–542	—	—
hK2 and IS-hK2 3' primer	G	(modC)CCCAGAATCACCCACAA [¶]	666–684	Biotin	1
PSA and IS-PSA 5' primer	H	TGAACCAGAGGAGTTCTTGAC	523–543	—	—
PSA and IS-PSA 3' primer	I	(modC)CCCAGAATCACCCGAGCAG	667–685	—	—
PSA and IS-PSA 3' primer	J	(modC)CCCAGAATCACCCGAGCAG	667–685	Biotin	1
Hybridization assays					
hK2 probe	K	(modC) ₂₀ GCTAGAGCTTACTCTGA	595–611	Eu ³⁺	11
IS-hK2 probe	L	(modC) ₂₀ GCTAGATTACTCTGAG	595–600 603–612	Tb ³⁺	11
hK2 target DNA	M	(modC)CCTTCTCAGAGTAAGCTCTAGCACACA	590–616	Biotin	1
IS-hK2 target	N	(modC)CCTTCTCAGAGTAATCTAGCACACATG	589–600 603–616	Biotin	1
PSA probe	O	(modC) ₂₀ GCGCAAGTTCACCCTCA	596–612	Eu ³⁺	17
IS-PSA probe	P	(modC) ₂₀ GTGCGCAATCACCTC	594–601 604–611	Tb ³⁺	22

*Nucleotide positions of the oligonucleotides are based on hK2 and PSA mRNA sequences published by Riegman et al³⁷ and Lundwall et al³⁵, respectively.

[†]—, no label.

[‡]Italic letters denote bases that are not complementary to the target. Bold letters denote bases that serve as detection and cleavage sites for *EcoRI* in the primer A and *KpnI* in the primer B.

[§]The 5' primer for the construction of IS-hK2 was the same primer (A) that was used for the construction of pGEM3-hK2.

[¶]modC, diaminohexanedeoxycytidine.

0.4 μ mol/L 5' primer A; 0.4 μ mol/L 3' primer B; 200 μ mol/L each dNTP (Pharmacia Biotech, Uppsala, Sweden); and 2.5 U of *Pfu* DNA polymerase (Promega Corporation, Madison, WI). The amplification was carried out with Perkin-Elmer Cetus DNA Thermal Cycler using the following program: initial denaturation of 5 minutes at 95°C followed by 35 cycles of 95°C for 30 s, 46°C for 2 minutes and 72°C for 2 minutes 30 s. A final extension of 5 minutes at 72°C was carried out after the 35 amplification cycles. The amplified cDNA insert was cloned into the *EcoRI* and *KpnI* sites of the plasmid vector, pGEM3 (Promega Corporation, Madison WI), and the constructed pGEM3-hK2 plasmid was transformed into *Escherichia coli* XL2 Blue cells (Stratagene, La Jolla, CA). The sequence of hK2 cDNA was confirmed by nucleic acid sequencing of the plasmid.

For the preparation of the IS-hK2 cDNA construct, 2 bp (nucleotides 601–602 from the hK2 sequence) were deleted from pGEM3-hK2 cDNA plasmid using PCR primers A, C, D and E. The deletion was introduced with a gene splicing by overlap extension technique³⁶ that we used also previously to construct the pGEM-IS-PSA⁹ from the pGEM-PSA plasmid template.³⁵ The pGEM3-IS-hK2 plasmid was transformed into *E. coli* XL-2 Blue cells (Stratagene, La Jolla, CA, USA) and the 2-bp deletion was confirmed by nucleic acid sequencing of the hK2 sequence.

In Vitro Production and Purification of hK2, IS-hK2, PSA, and IS-PSA mRNA

In vitro production of the calibrator (hK2 and PSA) and IS (IS-hK2 and IS-PSA) mRNA was carried out with AmpliScribe T7 transcription kit (Epicentre Technologies, Madison, WI). Linearized pGEM3-hK2, pGEM3-IS-hK2, pGEM3-PSA, and pGEM3-IS-PSA plasmids served as templates in the transcription, and the *in vitro* mRNA productions were purified as described previously.⁹ The purified mRNA pellet was dissolved in diethylpyrocarbonate (DEPC) treated water and stored in aliquots at –70°C. The amount of the mRNA was quantified using RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands) and the quality of the mRNA was checked by agarose gel electrophoresis. The pure mRNA samples were used to optimize the RT-PCR amplification, to control variations during the sample analyses (from the beginning of the RNA extraction to the detection of amplification products), and to generate calibration curves for the multiplexed PSA and hK2 RT-PCR assay.

Total RNA Extraction

Total RNA was extracted from the pelleted LNCaP, SP2/O, and blood nucleated cells using a TRIZOL reagent (Life

Technologies, Inc., Grand Island, NY). The RNA extraction was performed as described earlier.²⁴ A constant amount of IS-hK2 and IS-PSA mRNA (5×10^4 molecules of each) were added into each sample after denaturation of the pelleted cells. Samples containing only 2.5×10^6 SP2/0 cells were put up to serve as negative controls in RNA extraction. Denatured LNCaP and SP2/0 cells were combined to have a calculated amount of 1, 5, 10, 50, 100, 500 and 1000 denatured LNCaP cells in 2.5×10^6 SP2/0 cells. The RNA samples were stored at -70°C until they were analyzed.

RT-PCR Amplification

The cDNA synthesis was carried out with First-Strand cDNA Synthesis Kit using the *NotI*-d(T)₁₈ primer (Amersham Pharmacia Biotech AB, Uppsala, Sweden). In addition to the reactions for the samples, each cDNA synthesis contained reactions for the calibration curve that was built using the *in vitro* produced and purified calibrator and IS mRNAs diluted in an inert carrier solution of 0.2 g/L *E. coli* tRNA (Boehringer Mannheim GmbH, Mannheim, Germany). This carrier solution was also used as a sample in the negative control reaction for the cDNA synthesis. The cDNA synthesis was performed in a final reaction volume of 15 μl . After the synthesis a 7.5- μl cDNA sample was amplified in a 100- μl PCR reaction, which consisted of 10 mmol/L Tris-HCl, pH 8.8; 50 mmol/L KCl; 1 ml/L Triton X-100; 3.5 mmol/L MgCl_2 ; 400 $\mu\text{mol/L}$ each dNTP (Pharmacia Biotech, Uppsala, Sweden); and 1 U DynaZyme II recombinant DNA polymerase (Finzymes Oy, Espoo, Finland). The concentrations of the primers were 0.15 $\mu\text{mol/L}$ of primer F, and 0.15 $\mu\text{mol/L}$ of biotinylated primer G for the amplification of hK2 and IS-hK2, and 0.2 $\mu\text{mol/L}$ of primer H, 0.065 $\mu\text{mol/L}$ unlabeled primer I, and 0.035 $\mu\text{mol/L}$ biotinylated primer J for the amplification of PSA and IS-PSA. Before amplification the PCR reactions were kept on ice. The PCR amplification was performed in a PTC-200 DNA Engine (MJ Research, Inc., Watertown) using a program of 94°C for 30 s (2 minutes for the first cycle), 62°C for 30 s, and 72°C for 45 s (10 minutes 45 s for the last cycle) for 30 cycles.

Solution Hybridization

The products of the PCR amplification were analyzed by dual-label hybridization assay based on time-resolved fluorometry (TRF). The hybridization was performed as reported previously.²⁴ Briefly, a 10- μl aliquot of PCR product and 50 μl of buffer containing 1 mol/L NaCl were added into each streptavidin-coated microtitration well (InnoTrac Diagnostics Oy, Turku, Finland). Each PCR product was added into two wells and the target (PSA or hK2) and IS (IS-PSA or IS-hK2) products were detected from the same wells with the Eu^{3+} -labeled target and Tb^{3+} -labeled IS probes, respectively. PCR amplification and the biotinylated 3' primer (G or J) resulted in biotinylated PCR products, which were captured onto the streptavidin-coated microtitration wells by incubating at

room temperature with slow shaking for 30 minutes. After the capture reaction, the wells were washed three times with wash solution (PerkinElmer Life Sciences, Wallac Oy, Turku, Finland), and 100 μl of 50 mmol/L NaOH was added into each well for the denaturation of the double-stranded PCR products. Denaturation was carried out by incubating at room temperature with slow shaking for 5 minutes, the denatured DNA strand was then removed by washing three times as described above. The captured DNA strand was detected by adding 100 μl of hybridization solution containing detection probes, nonfat milk powder, and 1 mol/L NaCl. The hybridization solution for the detection of hK2 and IS-hK2 contained 0.05% nonfat milk powder, and 20 pg/ μl of both the hK2 and IS-hK2 detection probes (K and L), and the solution for the detection of PSA and IS-PSA contained 0.1% nonfat milk powder, 20 pg/ μl of the PSA probe (O), and 10 pg/ μl of the IS-PSA probe (P). After hybridization at $+40^\circ\text{C}$ (hK2, IS-hK2) or $+50^\circ\text{C}$ (PSA, IS-PSA) for 2 hours the wells were washed six times with $+40^\circ\text{C}$ (hK2, IS-hK2) or $+55^\circ\text{C}$ (PSA, IS-PSA) wash solution. Then 200 μl of enhancement solution for Eu^{3+} (PerkinElmer Life Sciences) was added into each well and after shaking for 30 minutes at room temperature the Eu^{3+} fluorescence was measured with 1420 Victor™ Multilabel Counter (PerkinElmer Life Sciences). After the measurement of the Eu^{3+} fluorescence, 50 μl of enhancement solution for Tb^{3+} (PerkinElmer Life Sciences) was added into each well and after a 5 minute incubation the Tb^{3+} fluorescence was measured.

Results

pGEM3-hK2 and pGEM3-IS-hK2

The hK2 cDNA sequence in the pGEM3-hK2 plasmid was confirmed for the lack of secondary mutations possibly originating from the PCR amplification with *Pfu* DNA polymerase and for the lack of alternatively spliced hK2 form.³⁷ The hK2 cDNA was found to correspond the correct hK2 mRNA sequence encoding the previously published 237-amino-acid protein.³⁸ Furthermore, the pGEM3-IS-hK2 plasmid was confirmed to contain the desired 2-bp deletion in the middle of the binding areas of the hK2 and IS-hK2 hybridization probes. The pGEM3-hK2 and pGEM3-IS-hK2 constructs were used, in combination with the pGEM3-PSA³⁵ and pGEM3-IS-PSA⁹ constructs, as templates to produce mRNA (see Materials and Methods) for the calibration and validation of the multiplexed hK2 and PSA RT-PCR assay.

Assay Design

In the assay developed, the basis for the quantification of the target mRNAs (hK2 and PSA) in the cell pellet is the use of an external calibration curve in RT-PCR and the addition of the target-like IS mRNAs (IS-hK2 and IS-PSA) to the samples at the beginning of the RNA extraction. The principle of the assay is shown in Figure 1 (A and B). Compared with the wild-type hK2 and PSA, the IS-hK2

and IS-PSA mRNAs contain a 2-bp deletion in the middle of the binding area of the detection probes for the PCR products. The calibration curve covering the range of $50\text{--}10^6$ copies of hK2 and PSA mRNAs contain a constant number of IS-hK2 and IS-PSA mRNAs (5000 copies) mixed with the varying number of the calibrator (target) mRNAs. For the quantification of hK2 and PSA mRNAs in a sample, a constant number of IS-hK2 and IS-PSA mRNA copies (50,000 copies, i.e., 10 times more than in the calibration curve) are added to the sample at the beginning of the RNA extraction. The RNA pellet is dissolved into $80\text{ }\mu\text{l}$ of sterile RNase-free water and 10% (8 μl) of each sample (corresponding to 5000 copies of IS-PSA and IS-hK2 mRNAs in theory) is analyzed with the RT-PCR assay. The target and the IS mRNAs in the sample and in the external calibration curve are co-amplified by RT-PCR in the same amplification mixture (Figure 1A). The cDNA synthesis is carried out using an universal oligo-d(T)₁₈-primer and the PCR mixture contains two specific primer pairs, one for the amplification of hK2 and IS-hK2 and the other for the amplification of PSA and IS-PSA. The PCR conditions will selectively produce hK2 and IS-hK2 or PSA and IS-PSA based on the presence of the template. After the 30-cycle PCR, the amounts of the target and IS amplification products are quantified by dual-label solution hybridization assays and TRF using specific Eu^{3+} and Tb^{3+} chelate labeled detection probes for the target and the IS, respectively (Figure 1B). The amount of the target mRNA in the sample can be then calculated by comparing the background-corrected target-to-IS fluorescence ratio in the sample to the ratio in the calibration curve. In addition, the use of the IS-hK2 and IS-PSA mRNAs allows the control of variations during the QRT-PCR assay from the RNA extraction to the detection of amplification products by solution hybridization.

To obtain a reliable quantification of the target mRNA by QRT-PCR with exogenous IS, there are at least two main factors to consider in the development of the assay: equal amplification efficiency of the target and the corresponding IS, and means of specific detection of the target and IS amplification products. We have previously shown that PSA and IS-PSA are amplified with the equal efficiency, and that the PSA and IS-PSA amplification products are detected without cross-hybridization.⁹ The amplification efficiencies of hK2 and IS-hK2 were studied by amplifying 1000 mRNA copies of each for different number of amplification cycles (Figure 2). It has been shown that if the initial amount of target (N_i), number of

Figure 1. Schematic presentation of the principle of the multiplexed QRT-PCR assay for the detection of hK2 and PSA mRNAs. **A:** A known amount of IS-hK2 and IS-PSA mRNAs are added to the denatured nucleated blood cells before total RNA extraction. PSA and hK2 mRNAs in the sample and the IS-hK2 and IS-PSA mRNAs are co-amplified by RT-PCR. A PCR reaction contains specific primers for the amplification of the hK2 (and IS-hK2) and PSA (and IS-PSA). Biotinylated 3' primers generate biotinylated amplification products that are captured on streptavidin-coated microtitration wells. **B:** The amplification products are detected by specific hybridization so that the hK2 and IS-hK2 products are detected in one well and the PSA and IS-PSA products in the other. The target specific hybridization probes are labeled with Eu^{3+} -chelate and the IS probes with Tb^{3+} -chelate. BIO, biotin; SA, streptavidin.

Figure 2. Amplification efficiency of hK2 and IS-hK2. Fluorescence signals obtained from amplification of 10^3 copies of hK2 (square) and IS-hK2 (triangle) mRNAs for different number of cycles.

amplification cycles (c), and amplification efficiency (f) are known, the amount of amplification product (N_c) can be calculated using the equation $\log N_c = \log N_i + c[\log(1 + f)]$.³⁹ Furthermore, in a plot of $\log N_c$ versus cycle number the slope of the curve equals to $\log(1 + f)$. In other words, the amplification efficiency of the target can be calculated using the described plot and equation.

In Figure 2 ($\log N_c$ versus cycle number), the slopes for hK2 and IS-hK2 are 0.27 that results in the same amplification efficiency for both the targets, $f = 0.86$. This means that the amount of amplification product increases 86% during each cycle when the PCR is in its exponential phase. The hK2 and IS-hK2 amplification products were detected by the optimized dual-label solution hybridization assay (see Materials and Methods) without cross-hybridization of the hK2 probe to the IS-hK2 product or IS-hK2 probe to the hK2 product.

In the case of assays where the RT-PCR amplification of multiple analytes is needed, the amplification and detection of the amplification products should be specific only for the right target(s) to be amplified and detected. In other words, hK2 or IS-hK2 should not be amplified by the PSA primers, and binding of PSA or IS-PSA probes to hK2 or IS-hK2 amplification products should not occur, and *vice versa*. The assay conditions were optimized to obtain the highest efficiency in RT-PCR and in detection of the amplification products by hybridization (see Materials and Methods). We studied the combined PSA and hK2 PCR by amplifying samples containing PSA, IS-PSA, hK2, and IS-hK2 using only the hK2 primers, only the PSA primers, or both hK2 and PSA primers in the reaction mix. The Figure 3 (A and B) shows that the result is the same regardless of amplification of the target and IS by PCR containing only the specific set of primers or both hK2 and PSA primers. In addition, non-specific amplification was not detected, the hK2 primers amplified only hK2 and IS-hK2, and the PSA primers only PSA and IS-PSA. Furthermore, hK2 and IS-hK2 detection probes did not yield signal from PSA and IS-PSA samples, neither did PSA and IS-PSA probes from hK2 and IS-hK2 samples.

Figure 3. Comparison of multiplexed and separate RT-PCR reactions. **A:** Amplification of different number of hK2 mRNA ($50-10^6$ copies) with a constant number of IS-hK2 mRNA (5000 copies) by PCR containing only hK2 specific primers (diamond) and both hK2 and PSA specific primers (square). **B:** Amplification of PSA mRNA ($50-10^6$ copies) with a constant number of IS-PSA mRNA (5000 copies) by PCR containing only PSA specific primers (diamond) and both PSA and hK2 specific primers (square).

Figure 4. Calibration curves of the developed multiplexed QRT-PCR assay for hK2 and PSA. **A:** Within- and between-assay variation over the calibration curve for hK2 mRNA generated with a constant amount of IS-hK2 mRNA (5000 copies). **B:** Within- and between-assay variation over the calibration curve for PSA mRNA generated with a constant amount of IS-PSA mRNA (5000 copies). Within-assay variation is presented with closed and open diamonds indicating the calibration curve and its CV%, respectively. Between-assay variation is presented with closed and open triangles indicating the calibration curve and its CV%.

Detection Limit and Reproducibility of the Multiplexed hK2 and PSA Assay

A calibration curve was generated with dilutions containing various number of hK2 and PSA mRNAs mixed with a constant amount of 5×10^3 of IS-hK2 and IS-PSA mRNAs. The analytical detection limit (2 times the mean of background signal) of the multiplexed assay developed was 50 hK2 and PSA mRNA copies in RT-PCR with a linear detection range up to 10^6 hK2 and PSA mRNA copies (Figure 4). The within- and between-assay reproducibility was determined with the calibrators (50 – 10^6 hK2 and PSA mRNA, 5×10^3 IS-hK2 and IS-PSA in each dilution) using two replicas of each sample in five independent experiments. The within-assay CVs over the calibration range were 30.8–3.5% for the hK2 calibrators (Figure 4A) and 21.7–1.4% for the PSA calibrators (Figure 4B). The corresponding between-assay CVs were 31.4–5.1% and 34.2–4.4% for the hK2 (Figure 4A) and PSA (Figure 4B) calibrators, respectively. Within the functional working range of 10^2 – 10^6 hK2 and PSA mRNA copies the within-assay CVs were under 11% and the between-assay CVs were under 23%.

hK2 and PSA mRNAs in LNCaP Cells

The assay was validated from the RNA extraction level with samples containing 1 – 10^3 LNCaP cells in 2.5×10^6 hK2- and PSA-negative SP2/0 cells. The number of hK2 and PSA mRNA copies in LNCaP cells were studied using six replicas of each LNCaP dilution in three independent experiments. The overall mean number of hK2 mRNA copies in 10 – 10^3 LNCaP cells ranged from 3700

to 129,000 (Figure 5A). The mean number of hK2 mRNA copies per one LNCaP cell was 230 ± 110 . The mean number of PSA mRNA copies in 1 – 10^3 LNCaP cells ranged from 1500 to 494,000 (Figure 5B). The mean number of PSA mRNA copies per one LNCaP cell was 630 ± 290 . Using the multiplexed QRT-PCR assay it was possible to detect 1 PSA-producing and 10 hK2-producing LNCaP cells in the presence of 2.5×10^6 hK2- and PSA-negative SP2/0 cells.

hK2 and PSA mRNAs in Blood Samples

The multiplexed QRT-PCR assay was further validated with blood samples from 25 PC patients and 16 healthy controls. The RNA samples were analyzed twice to confirm the results. The samples with undetectable hK2 or PSA mRNA were assigned a level of 100 copies (i.e., below the functional detection limit) in statistical analyses of the data. Neither PSA nor hK2 mRNAs were found in healthy women ($n = 9$) and healthy men ($n = 7$) controls. PSA and hK2 mRNA expression was detected in 20 of 25 (80%) and 19 of 25 (76%) PC patients, respectively. All of the patients that gave detectable hK2 mRNA levels gave also detectable PSA mRNA. The median number of PSA and hK2 mRNA copies were 4300 and 21,000 in patients with biochemically progressive PC (group A, $n = 4$), 4800 and 53,000 in hormonally treated patients with locally advanced and metastasized PC (group B, $n = 13$), and 100 for both PSA and hK2 in patients with rapidly progressive hormone refractory PC and multiple metastases (group C, $n = 8$). Compared to the PSA mRNA copies, the number of hK2 mRNA copies was significantly higher in patient groups A ($P = 0.02$) and B ($P = 0.004$) (Mann-

Figure 5. Number of hK2 and PSA mRNA copies in LNCaP cells. **A:** Mean number of hK2 mRNA in 10–1000 LNCaP cells. **B:** Mean number of PSA mRNA in 1–1000 LNCaP cells. Error bars show the SD of three independent experiments. Each experiment included six replicates of each cell dilution.

Whitney *U* test). However, one patient in group B gave 29% higher number of PSA mRNA than hK2 mRNA. Interestingly, in patient group C only 2 of 8 and 3 of 8 patients gave detectable (i.e., 100 mRNA copies in RT-PCR) hK2 and PSA mRNA expression in blood samples, respectively. Of the patients in group C, only one patient gave 47% higher number of hK2 mRNA than PSA mRNA, in addition, one patient gave 4% higher number of PSA mRNA than hK2 mRNA, and one patient gave detectable PSA mRNA but no hK2 mRNA. Serum total PSA values were available for all of the PC patients except for one patient in group B and for two patients in group C. The median serum total PSA values in the three patient groups were 19 ng/ml for group A, 379 ng/ml for group B and 257 ng/ml for group C. Serum PSA values did not correlate with the number of PSA and hK2 mRNA copies. No serum total PSA values were available for the healthy controls. Box plot presentations of number of hK2 and PSA mRNA copies measured by the multiplexed QRT-PCR assay in the different patient groups are given in Figure 6. With the results of this small patient material, a Kruskal-Wallis test showed that hK2 and PSA can discriminate the three patient groups from each other, $P = 0.0002$ and $P = 0.004$, respectively. However, there is significant overlap with the mRNA copy numbers in patient groups A and B. The power of PSA mRNA, hK2 mRNA and serum total PSA to discriminate the three patient groups and controls is shown in Table 2.

Discussion

We described here a development of a multiplexed QRT-PCR assay using TRF-based solution hybridization for the detection of PSA and hK2 mRNAs in peripheral blood of PC patients. Compared to two separate assays, the multiplexed assay developed offers a tool for a simultaneous

quantification of the hK2 and PSA mRNAs from the same patient sample, and allows reproducible, specific, sensitive, more rapid, and less expensive detection of the two targets. The assay uses two exogenous IS mRNAs, IS-hK2 and IS-PSA that differ only by a 2-bp deletion from

Figure 6. Box plot representation of the hK2 and PSA mRNA copies in blood samples from healthy women (F, $n = 9$) and men (M, $n = 7$) controls and from PC patients obtained with the multiplexed QRT-PCR assay for hK2 and PSA. All of the controls gave result below the functional detection limit of 100 mRNA copies. The patients included 4 subjects with biochemically progressive PC (group A), 13 subjects with locally advanced and metastasized PC under hormonal treatment (group B), and 8 subjects with rapidly progressive PC and multiple distant metastases (group C). Patients from group C with number of hK2 and PSA mRNA copies below the functional detection limit were assigned a level of 100 copies in the statistical analysis of the data. The vertical lines of the box plots represent the 10th, 25th, 50th, 75th, and 90th percentiles.

Table 2. The *p* Values Obtained by Mann-Whitney *U* Test after Comparison of PSA mRNA, hK2 mRNA and Serum Total PSA Values of Various Patient Groups and Healthy Controls

Analyte	Patient groups	A	B	C
PSA mRNA	A			
	B	NS*		
	C	0.0415 [†]	0.0014	
hK2 mRNA	Controls	0.0025	<0.0001	NS
	A			
	B	NS		
Serum total PSA	C	0.0066	0.0002	
	Controls	0.0025	<0.0001	NS
	A			
	B	0.029		
	C	0.05	NS	
	Controls	— [‡]	—	—

*NS, not significant.

[†]A *p* value equal to 0.05 or less was considered significant.

[‡]—, serum total PSA values were not available for the controls.

Group A, biochemical progression; group B, metastatic; group C, metastatic, hormone refractory.

their target mRNAs (hK2 and PSA). A known amount of the synthetic IS-hK2 and IS-PSA mRNAs are added into each collected and denatured cell pellet at the beginning of the RNA extraction to enable a standardization correcting for any variations starting from the beginning of the RNA extraction to the final detection of the amplification products by solution hybridization. In addition, the use of an external calibration curve, consisting of the *in vitro* produced and purified hK2, PSA, IS-hK2, and IS-PSA mRNAs, allows the quantification of hK2 and PSA mRNA copies in the sample by comparing the target-to-IS fluorescence ratio of the sample to the ratio in the calibration curve.

After the RNA extraction, the target and the IS mRNAs in the samples and calibrator reactions were co-amplified by RT-PCR. Instead of using a large number of amplification cycles and nested-PCR approach, we used an optimized 30-cycle PCR and two pairs of primers to specifically amplify hK2 and IS-hK2 cDNAs with one pair and PSA and IS-PSA cDNAs with the other pair in the same amplification reaction. Thereafter, the amplification products were detected by dual-label solution hybridization based on TRF technology in two separate microtitration wells. The TRF offers a sensitive, nonradioactive, and rapid method for the quantification of the amplification products.⁴⁰ The QRT-PCR assay developed gave an analytical detection limit of 50 (i.e., 2 times the mean of background signal) and a functional detection limit of 10² (i.e., between-assay CVs under 23%) target mRNA copies with a linear range up to 10⁶ mRNA copies.

We evaluated the multiplexed QRT-PCR assay on cultured LNCaP cells and samples from PC patients (*n* = 25) and healthy volunteers (*n* = 16). Based on the Northern blot analyses of PSA and hK2 mRNAs using non-cancerous prostatic tissue samples, it has been reported that the level of hK2 mRNA expression relative to that of PSA mRNA is approximately 10 to 20%.⁴¹ In the analysis of LNCaP cells, we showed that the mean hK2 mRNA expression relative to the mean PSA mRNA expression was

28.8 to 36.5%. In our previous reports we have found that the mean number of PSA mRNA per one LNCaP cell ranged from 500 to 2100 copies⁹ and from 670 to 1100 copies.²⁴ In this paper we found the mean number of PSA mRNA per one LNCaP cell was from 340 to 920 copies, which is slightly smaller than the results obtained previously. Despite the identical culture conditions, this variation in the number of PSA mRNA copies is most probably due to normal batch variation in LNCaP cell cultures. Furthermore, some of the variations may be due to difficulties in diluting very small amounts of LNCaP cells (1–10 cells), which affects the final calculated number of target copies in the sample. These issues in turn demonstrate how important it is to develop RT-PCR assays that do not use LNCaP cells as a calibrator material. Conversely, properly calibrated QRT-PCR assays should be used as tools to study the different levels of target mRNA expression in the cultured cells.

There are four previously published reports on qualitative hK2 RT-PCR assays for the detection of PC cells in patient samples. Young et al.¹² were the first to report the detection of hK2 mRNA together with that of PSA mRNA by two qualitative RT-PCR assays.^{7,12} They detected hK2 mRNA in 1 of 1 PC patient with prostate-confined disease, 2 of 3 patients with locally advanced disease and 2 of 2 patients with distant metastatic disease. PSA mRNA was detected only in 1 of 2 patients with distant metastatic disease. However, they did not describe the experimental details of their hK2 assay. It remained unclear, for example, what were the sequences of the PCR primers and whether the hK2 assay detected only hK2 and not PSA mRNA. Neither PSA nor hK2 were detected in the controls. Corey et al.^{8,10} used two separate RT-PCR assays to detect hK2 and PSA from both the peripheral blood and bone marrow samples from patients with advanced PC (*n* = 13) and patients with clinically localized disease (*n* = 63). All blood and bone marrow samples from controls were found to be negative for hK2 and PSA. In blood samples from patients with clinically localized PC, detectable PSA and hK2 mRNAs were found in 12 of 63 and 8 of 63, respectively. In blood samples from patients with advanced PC, 6 of 13 were positive for PSA and 4 of 13 were positive for hK2. These results showed that the patient samples were more often positive for PSA mRNA than for hK2 mRNA. Corey et al.^{8,10} detected PSA and hK2 mRNAs also in bone marrow samples from the same patients. They found that the number of patients that gave positive result for PSA and hK2 mRNAs was higher in the bone marrow samples than in the blood samples. Kawakami et al.¹¹ studied expression of hK2 and PSA mRNA in blood samples from PC patients (*n* = 41) and controls. Neither PSA nor hK2 amplification products were found in the control samples from 20 healthy volunteers and 7 patients with benign prostatic hyperplasia. They detected PSA mRNA in 6 of 7 patients with nonpalpable cancer, 2 of 5 patients with prostate-confined disease, 4 of 8 patients with locally advanced disease, 3 of 4 patients with pelvic lymph node involvement, and 14 of 17 patients with distant metastatic disease. Of all of the PC patients, hK2 mRNA was found only in 7 of 17 patients with distant metastatic disease. Based on

their finding that hK2 mRNA was only found in patients with distant metastatic PC, Kawakami et al¹¹ concluded that hK2 mRNA is associated with the metastatic progression of PC and is an indicator of a poor prognosis. Slawin et al⁴² developed a splice variant-specific RT-PCR assay for hK2 mRNA and evaluated the assay on blood samples from healthy men ($n = 14$), patients with metastatic PC ($n = 7$) and patients undergoing radical prostatectomy for clinically localized PC ($n = 228$). The assay detected two forms of hK2 mRNA: the native transcript, which encodes for the full-length hK2 protein, and an alternatively spliced transcript, which contains 37 additional nucleotides downstream from the native splice donor site in intron IV.³⁷ The native hK2 mRNA and the alternatively spliced hK2 mRNA were detected in 57 of 228 and 58 of 228 patients undergoing radical prostatectomy, respectively. The results showed that the preoperative expression of the native hK2 but not the alternatively spliced hK2 mRNA added prognostic information to the prediction of lymph node-positive disease in patients undergoing radical prostatectomy. Native and alternatively spliced hK2 mRNAs were detected in 5 of 7 and 1 of 7 patients with metastatic PC, respectively. However, native and alternatively spliced hK2 mRNAs were also detected in 2 of 14 and 5 of 14 healthy controls, respectively.

Interestingly, we found that the number of hK2 mRNA copies was significantly higher than the number of PSA mRNA copies in PC patients with biochemically progressive disease (group A, $n = 4$, $P = 0.02$) and with locally advanced and metastasized disease (group B, $n = 13$, $P = 0.004$). It was also found that hK2 mRNA expression was lower than PSA mRNA expression only in two patients that gave detectable result. One patient with locally advanced and metastasized PC had 29% hK2 mRNA expression relative to PSA mRNA expression, and the other patient with hormone refractory and distantly metastatic PC had 4% hK2 mRNA expression relative to PSA mRNA expression. Furthermore, the preliminary results showed that the number of hK2 and PSA mRNA copies differentiated the patients with biochemically progressive disease and with advanced disease from the patients with fast progressive hormone refractory disease and from the controls.

Our results showed that the overall number of patients that gave positive result for PSA mRNA (80%) was higher than that for hK2 mRNA (76%). This result is in accordance with the results obtained by Corey et al¹⁰ and Kawakami et al¹¹ but not with the results of Young et al.¹² The patients that gave positive result for hK2 mRNA were also positive for PSA mRNA. Only in patients with hormone refractory PC, we found one patient who expressed PSA mRNA in blood while no hK2 mRNA was detected. However, contrary to the previous reports,^{10–12} we did not find patient samples that would have given detectable hK2 mRNA and no PSA mRNA. This may be due to the small number of patient samples in the preliminary evaluation of the assay.

The results of Young et al¹² showed hK2 and PSA mRNA expression in 83% and 17% of all of the patients studied, respectively. It was surprising that PSA mRNA

was not detectable in the blood samples from the patients with organ-confined and with locally advanced PC. Our assay detected PSA and hK2 mRNA copies in 100% of the blood samples from the patients with the biochemically progressive PC (no metastases detected) and with the advanced PC. However, only 38% and 25% of the patients with hormone refractory PC were positive for PSA and hK2 mRNA, respectively. Corey et al¹⁰ detected PSA and hK2 in 19% and 13% of the patients with clinically localized PC, and in 46% and 31% of the patients with metastatic PC, respectively. Furthermore, Slawin et al⁴² detected the expression of the native hK2 mRNA in 23% of the patients with organ-confined PC, 30% of the patients with locally advanced PC, 71% of the patients with distant metastatic PC, and 14% of the healthy men controls. Kawakami et al¹¹ found PSA mRNA in 67% of the patients with organ-confined PC, 58% of the patients with advanced PC, and 82% of the patients with distant metastatic PC, whereas hK2 mRNA was found only in 41% of the patients with distant metastatic PC. From the patients with distant metastatic PC, 4 were refractory to the hormonal therapy. PSA and hK2 mRNAs were found in 3 of 4 (75%) and 4 of 4 (100%) of these patients, respectively. Contrary to the results of Kawakami et al, we found decreased expression of PSA and hK2 mRNA in the patients with hormone refractory PC. The reason for the high number of hK2 and PSA negative results may owe to the late stage of the disease of these patients. It must be noted, however, that these results derive from a small number of patients. Further studies with a larger number of samples and long-term follow-up will be required to confirm this phenomenon. However, the differences in the results may also be due to the various assay concepts and PCR primers, which result in amplification products from different forms of PSA and hK2 mRNAs.

Both PSA and hK2 mRNA transcripts are present in several alternatively spliced forms.^{37,43–45} However, very little is known about the presence or function of the products encoded by these alternative mRNAs. At least four PSA and five hK2 mRNA forms have been characterized. Riegman et al⁴⁵ characterized three of the PSA transcripts. The major 1.5 kb PSA mRNA encodes for the native PSA protein. Additional forms of PSA transcripts included a truncated 0.9 kb mRNA with 145 additional nucleotides from the intron III between the exons 2 and 3, and a 1.9 kb mRNA with 442 additional nucleotides from the intron IV between the exons 4 and 5. Recently, Heuzé et al⁴³ described a 2.1 kb alternative PSA mRNA with 644 additional nucleotides from the intron IV between the exons 4 and 5. Riegman et al³⁷ reported that in addition to the native 1.5 kb hK2 mRNA, there is an alternatively spliced variant, which contains 37 additional nucleotides from the intron IV between the exons 4 and 5. More recently, Liu et al⁴⁴ reported three additional species of hK2 transcripts, including two 3.0 kb transcripts corresponding to the two above mentioned 1.5 kb hK2 isoforms, but each with an additional 1.5 kb of untranslated region attributable to transcription through the first polyadenylation signal to a second signal located downstream. The third 1.5 kb transcript contains a deletion of 13 nucleotides between exons 3 and 4 of the hK2 mRNA.

The different assays seem to detect different forms of PSA and hK2 transcripts. The PSA and hK2 primers of our assay were designed so that the 5' primers spanned the junction between the exons 3 and 4, and the 3' primers spanned the junction between the exons 4 and 5. The PSA and hK2 PCR primers amplified a 163-bp fragment spanning the end of exon 3, exon 4, and the beginning of the exon 5. Therefore, the PSA primers produced the amplification product from the native 1.5 kb mRNA. The hK2 primers amplified the 163-bp fragment from the native 1.5 kb mRNA and its related 3.0 kb mRNA with the 1.5 kb 3' untranslated region. Neither the PSA nor the hK2 primers did amplify the other alternatively spliced forms of the transcripts. Young et al.¹² used in their study PSA primers that were previously developed by Katz et al.⁷ In addition to the native PSA mRNA, these primers amplified a fragment spanning exons 3 and 4 from the three above-mentioned alternative PSA mRNA forms. The sequences of the hK2 primers were not reported. Corey et al.¹⁰ used hK2 primers that amplified a fragment spanning exons 2 and 4, and PSA primers that produced a fragment spanning exons 2 and 5. Both the PSA and the hK2 primers allowed the amplification of all of the above-mentioned forms of PSA and hK2 transcripts. Kawakami et al.¹¹ used hK2 primers that amplified a fragment spanning exons 2 and 3. The primers for the PSA assay shared the same sequences as reported previously by others.¹³ The PSA primers amplified a fragment spanning exons 3 and 5. The PSA and hK2 primers used by Kawakami et al.¹¹ allowed also the amplification of all of the above-mentioned alternative PSA and hK2 transcripts. Slawin et al.⁴² studied the expression of the native hK2 mRNA and the alternatively spliced hK2 mRNA with the additional 37 nucleotides between the exons 4 and 5. The hK2 primers amplified a fragment spanning exons 4 and 5 so that they amplified both the native and the alternative transcripts and their related 3.0 kb mRNAs with the 1.5 kb 3' untranslated region.

Lately, the immunofluorometric studies on hK2 and PSA protein expression in serum of prostate cancer patients have shown that measurements of hK2 in addition to PSA have significantly enhanced the discrimination of PC patients from patients with benign prostate disease,⁴⁶ improved the identification of poorly differentiated prostate tumors,⁴⁷ and allowed more accurate prediction of organ-confined from nonorgan-confined disease.^{47,48} Furthermore, the studies on hK2 and PSA protein expression on tissue level have revealed that both hK2 and PSA are down-regulated in malignant tissue compared with normal prostate tissue.⁴⁹ The ultimate additional benefit of quantitative measurements of hK2 and PSA mRNA expression aiming to improve PC detection and molecular staging awaits continued investigations. A drawback of the RT-PCR assays is that they cannot distinguish whether the number of target mRNA copies indicates a few cells that contain many target mRNA molecules or many cells that contain a few target mRNA molecules. However, quantitative assays capable of detecting different levels of target mRNAs may prove to be useful in determining the clinically significant numbers of the target copies. If used in combination with cell sorting meth-

ods, quantitative RT-PCR assays may be valuable tools in solving the problem with the number of target mRNAs per cancer cell.

In conclusion, the multiplexed QRT-PCR assay developed provides sensitive and specific detection of the number of hK2 and PSA mRNA copies in blood samples. The quantification of PSA and hK2 mRNAs is based on the use of an external calibration curve consisting of target mRNAs and two target-like IS mRNAs. The IS mRNAs are also used to control the variations from the beginning of the RNA extraction to the detection of amplification products to allow reproducible quantification of the targets. To our knowledge, we are the first to report a multiplexed QRT-PCR assay for the simultaneous detection of hK2 and PSA mRNAs and for the quantification of an exact number of hK2 mRNA copies in LNCaP cells and in blood samples from PC patients. Further studies are needed to determine the impact of PSA and hK2 QRT-PCR in the molecular staging of PC.

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