Detection of Prostate Specific Transcripts in the Peripheral Blood During Brachytherapy Predicts Postoperative PSA Kinetics

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BACKGROUND. We evaluated whether detection of prostate-specific antigen (PSA) and human kallikrein 2 (hK2) transcripts in the peripheral blood during brachytherapy could predict biochemical outcome.

METHODS. Eighty-one patients who underwent 125Iodine-based brachytherapy for localized prostate cancer (Gleason score <8, PSA <20 ng/ml, stage <T3), participated in the study. Brachytherapy was given to 35 patients as monotherapy, to 36 in combination with androgen deprivation therapy (ADT), and to 10 in combination with external beam radiation and ADT. Blood samples from 80 patients were available for analysis. Nested RT-PCR means was used to detect mRNA expression of PSA and hK2 in the peripheral blood. Their expression was analyzed before, during and 1 month after brachytherapy. Patients’ biochemical outcome (blood PSA levels) during 3 years of follow-up was correlated with the PCR results.

RESULTS. The incidence of PSA and hK2 mRNA expression in the peripheral blood was significantly higher during than before or after the procedure. Patients with concurrent positive PSA and hK2 PCR results during brachytherapy had higher postoperative blood PSA values and a slower decline rate of PSA compared with patients with negative PSA and hK2 PCR results. No correlations were found between pre- and postbrachytherapy PCR results and biochemical outcome. ADT was the only significant factor that affected PSA and hK2 mRNA expression during brachytherapy.

CONCLUSIONS. Our short-term results suggest that detection of PSA and hK2 transcripts in the peripheral blood of prostate cancer patients during brachytherapy could serve as a predictor of biochemical outcome.

KEY WORDS: prostate cancer; brachytherapy; RT-PCR; peripheral blood; PSA; hK2

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of death among Western men of all ages [1,2]. Most men currently diagnosed with prostate cancer have an organ-confined disease. One of the treatment options of localized disease, besides surgery and external radiation therapy, is brachytherapy. Brachytherapy gained popularity among patients since the 1980s, having high quality implants with favorable treatment results [3–5]. Ultimately, the major risk faced by patients treated for organ-confined disease is recurrence and development of metastatic disease [6]. After brachytherapy,
biochemical (PSA) failure can occur because of local or distant recurrence [7]. In general, the rate of biochemical free from recurrence corresponds to disease risk measures, from low to high [7]. Still, a substantial number of low to intermediate risk patients with localized disease will experience recurrence after brachytherapy [7,8].

Reverse transcriptase polymerase chain reaction (RT-PCR) has been investigated as a potentially new diagnostic, staging and prognostic modalities for prostate cancer [9–11]. The expression of prostate-specific genes was found elevated in the peripheral blood during urologic procedure including radical prostatectomy, transurethral or open prostatectomy and prostate biopsy [12–16]. However, the clinical significance of these results is controversial and is still unclear. Herein, we used nested RT-PCR means to evaluate whether the expression of prostate-specific genes PSA and human kallikrein 2 (hK2) before, during and after brachytherapy in the peripheral blood of patients would predict biochemical outcome. Three-year results show that the presence or absence of simultaneous PSA and hK2 transcripts in the peripheral blood during brachytherapy could serve as a powerful factor to predict blood PSA values and kinetics during follow-up.

MATERIALS AND METHODS

Patients

Eighty-one of 145 patients who underwent brachytherapy in our center between November 27, 2003 and June 30, 2005 were included in the study after having signed an informed consent approved by the Institutional and National Supreme Helsinki Committees for Genetic Studies. All patients had clinically organ-confined prostate cancer with low to intermediate risk characteristics: PSA <20 ng/ml, Gleason score <8 and clinical stage <T3.

Seed Implantation

Brachytherapy, 125I permanent seed implantation was performed by using real-time intra-operative planning method as described previously [17,18]. Patients with a prostate gland larger than 50 ml were administered combined androgen deprivation therapy (ADT) for median of 6 months to reduce the gland size to a desired volume of <50 ml. Patients with a Gleason score of 7, independent of PSA levels and clinical stage, were treated with a combination of 125I seed implantation at a reduced dose (107 Gy) and external beam radiation (EBRT) (45 Gy). A 6-month ADT was prescribed to all patients treated with the combination. All patients underwent a computed tomography (CT)-based postimplant dosimetry evaluation at 1 month. Dosimetry parameters were calculated as described [17,18].

Follow-Up

All patients were seen and examined in the outpatient clinic at 1, 3, 6, 9, and 12 months after implantation and once yearly thereafter. Blood PSA was measured every 3 months during the first year and every 6 months thereafter. When relapse was suspected, either because of PSA elevation or because of clinical symptoms and/or signs, a systemic evaluation was performed, which included a bone scan, an abdominal CT scan, a chest X-ray and blood tests for complete blood count, renal and liver functions.

Isolation of Blood Samples and Total RNA Extraction

Two and a half ml of peripheral blood were collected into PAXgene tubes (PreAnalytiX PAXgene™ Blood RNA System, PreAnalytiX, Hombrechtikon, Switzerland; Qiagen, Hilden, Germany) before (at the time of anesthesia), during (immediately after needles have been inserted) and 1 month after brachytherapy (during the first visit at the outpatient clinic). Total RNA was extracted using PAXgene blood RNA isolation kit (PreAnalytiX; Qiagen). This method is concise and it reduces time consumption, obtaining high-quality RNA within 1 hr.

RT-PCR and Sample Analysis

We used commercially available standard kits to perform RT-PCR analyses (ABgene, Guildford, Surrey, UK). Nested PCR was utilized to detect low mRNA levels and to increase specificity of the prostate-specific markers, PSA and hK2. This method was proven efficient to detect prostate-specific mRNA even from a single prostate cell in a range of 10^5–10^8 peripheral blood mononuclear cells [19,20]. In each run of RT-PCR reactions we utilized LNCaP mRNA as a positive control and water as a negative control. Briefly, 1 μg RNA was reverse-transcribed from each sample. One and a half microliters of the RT product was subjected to PCR reaction using the external primers (Fig. 1). Subsequently, the first PCR product was diluted 1–50 and then 8 μl were subjected to the second (nested) PCR reaction using the internal primers (Fig. 1). Specific primer sequences were synthesized based on the sequences of each antigen sequences obtained from the GenBank. The specificity of the primers was examined by a BLAST search. To minimize false positive results, the expression of prostate-specific genes in the blood was considered positive only when two independent
tests of nested PCR reactions were positive. When equivocal results were obtained (one positive and one negative result) a third independent nested PCR reaction was performed. The mRNA expression was considered positive only when two out of the three nested PCR reactions were positive.

**Data Analysis**

Descriptive statistics, such as mean, standard deviation (SD), standard error of the mean (SE), range and proportion, were used to summarize the baseline characteristics of the patients. Two-way analysis of variance (ANOVA) using mixed model inter- and intra-subject design was used to analyze the primary endpoint results. With this model two independent variables were defined: time as repeated measures intra-subjects variable and groups categorized according to their status of PSA and/or hK2 mRNA in the peripheral blood as inter-subject variable. The interaction term when positive was further analyzed using post hoc LSD tests. Comparison among categorical variables was done using the $\chi^2$ test. Since eight time points (3, 6, 9, 12, 18, 24, 30, and 36 months) were compared among each two subgroups, Bonferroni correction was applied to determine the $P$-value cutoff that was set accordingly to 0.00625. The analyses were carried out using SPSS 15.0 (SPSS, Inc., Chicago, IL). All other tests were two-sided, and statistical significance was set at the level of $P$-value <0.05.

**RESULTS**

Patients’ characteristics are presented in Table I. Blood samples for analyses were available from 80 patients before and during brachytherapy and from 79 patients also after brachytherapy. Nested-PCR results of PSA and hK2 from selected patients, before, during, and after brachytherapy are demonstrated in Figure 2.

We analyzed all available samples for PSA and hK2 mRNA expression before, during and 1 month after brachytherapy (Table II). Before the procedure, PCR reactions were positive to PSA, to hK2 and to both PSA and hK2 (PSA/hK2) expression in 10%, 1.25% and none of the patients, respectively (Table II). The rate of positive PCR reactions to PSA, hK2, and PSA/hK2 had significantly increased during brachytherapy and was 31%, 22%, and 15% of the patients, respectively (Table II). Three patients had positive PCR to PSA before which remained positive during brachytherapy and three had positive PCR to PSA during which remained positive after brachytherapy. One patient had positive PCR to hK2 during brachytherapy which remained positive after the procedure. One month after brachytherapy, interestingly, none of the patients’ samples were positive for simultaneous PSA/hK2 expression (Table II).

Since the last patients participated in the study had only 3 years of follow-up we evaluated the biochemical outcome (blood PSA levels) during 3 years after brachytherapy. To assess whether the PCR results have impact on the biochemical outcome of the patients, we compared the blood PSA values and kinetics in patients who had positive PCR to those who had negative PCR before, during and after brachytherapy (Fig. 3). The most significant differences in the rate of blood PSA decrease and values were observed between patients with concurrent positive PCR to PSA/hK2 and patients...
with concurrent negative PCR to PSA/hK2 during brachytherapy (Fig. 3E). The calculated rate of PSA decline was 1.5-fold slower in patients with positive PSA/hK2 PCR than in patients with negative PSA/hK2 PCR (Fig. 3F). Similar results, but with a lesser significant extent, were also obtained between the patients with positive and negative PCR to PSA (Fig. 3B) and to hK2 during brachytherapy (Fig. 3D). There were no significant differences between the patients with positive and negative PCR to PSA before (Fig. 3A) or after brachytherapy (Fig. 3C).

Because ADT can affect blood PSA values and bias our PSA kinetics calculations and nadir values, we analyzed blood PSA kinetics among the different subgroups of patients as categorized in Table I: Group I, brachytherapy as monotherapy, without ADT at any time prior to, or within the period following brachytherapy (hormone naive); Group II, brachytherapy with ADT for prostate downsizing and Group III, brachytherapy combined with EBRT and ADT (Fig. 4A). Although, a different pattern of PSA decline was observed among the groups, the mean blood PSA was <1 ng/ml in all subgroups at 3 years of follow-up (Fig. 4A). The lowest mean PSA (0.032 ng/ml) was observed in Group III at 3 years of follow-up (Fig. 4A). No significant differences between patients with positive or negative PCR to PSA, hK2 and PSA/hK2, were observed in Group II and Group III during brachytherapy (data not shown). In contrast, in Group I (hormone naive patients), the rate of PSA decline was 2.4-fold slower in patients with positive PSA/hK2 PCR than in patients with negative PSA/hK2 PCR (Fig. 4E).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (range)</th>
<th>No. (%)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>68 (51–79)</td>
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<tr>
<td>Initial PSA (ng/ml)</td>
<td>7.7 (3.3–19.9)</td>
<td></td>
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<tr>
<td>Follow-up (months)</td>
<td>44 (36–54)</td>
<td></td>
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<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>55 (68)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>26 (32)</td>
<td></td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>71 (88)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10 (12)</td>
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<tr>
<td>Patients categorization</td>
<td></td>
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</tr>
<tr>
<td>Group I: Brachytherapy monotherapy(^a)</td>
<td>35 (44)</td>
<td></td>
</tr>
<tr>
<td>Group II: Brachytherapy with ADT(^b)</td>
<td>36 (44)</td>
<td></td>
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<tr>
<td>Group III: Brachytherapy with EBRT(^c)</td>
<td>10 (12)</td>
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\(^a\)Patients with Gleason score of ≤6 and prostate size ≤50 ml were treated with brachytherapy alone, without EBRT and without ADT at any time prior to, or within the period following brachytherapy (hormone naive).

\(^b\)Patients with Gleason score of ≤6 and prostate size >50 ml received a median of 6 months of ADT and were treated with brachytherapy.

\(^c\)Patients with Gleason score 7 were treated with combination of brachytherapy and EBRT and received a median of 6 months of ADT. PSA, prostate-specific antigen; EBRT, external beam radiation; ADT, androgen deprivation therapy.
To further emphasize the differences between the patients with positive versus negative PCR results we compared the portion of patients whose blood PSA was <0.5 ng/ml at each time point (Fig. 5). It can be seen that the number of patients (among all) with negative PCR to PSA or to PSA/hK2 had blood PSA level <0.5 ng/ml was significantly higher than the number of patients with positive PCR to PSA or to PSA/hK2 (Fig. 5A). This difference was even more prominent when patients in Group I were analyzed alone during the 3 years of follow-up (Fig. 5B). To evaluate the validity of the RT-PCR assays for PSA and PSA/hK2 during brachytherapy to predict blood PSA < or >0.5 ng/ml 3 years after brachytherapy the sensitivity, specificity, positive predictive value and negative predictive value were calculated (Table III). Including hK2 in the RT-PCR test increased specificity while it decreased sensitivity. However, in all groups both tests showed a high negative predictive value >0.9 (Table III).

Of interest, the percentage of patients with positive PCR to PSA and to PSA/hK2 during brachytherapy was significantly higher in Group I than in Groups II and III (Table IV). No other significant differences between patients with positive and negative PCR results were seen with other parameters including age, PSA values before treatment, clinical stage, Gleason score, number and specific activity of implanted seeds, prostate volume and dosimetric measures.

## DISCUSSION

RT-PCR has been shown to identify very small numbers of disseminated prostatic cells [21]. However, the biological and clinical significance of these cells is still debatable [22]. Some groups have found a significant association between preoperative detection of PSA mRNA in the peripheral blood and prostate cancer stage and progression while others have failed to show any significant correlations [9–11,23–34]. In this study, we utilized a sensitive nested RT-PCR methodology described by Hara et al. [19] to assess the clinical value of prostate specific gene expression in the peripheral blood during brachytherapy.

Several years ago, Siddiqua et al. [35] hypothesized that the insertion of multiple needles into the prostate gland during brachytherapy may provide a vascular access for prostate tumor cells to disseminate in the peripheral blood. Their results showed that the majority (65%) of 23 patients undergoing prostate brachytherapy turn positive for PSA mRNA expression during or after the procedure [35]. Moreover, PSA mRNA expression in the peripheral blood during and after brachytherapy significantly correlated with biochemical relapse during follow-up [35]. In our patients, there was no biochemical significance for the presence of PSA and/or hK2 mRNA in the peripheral blood before or 1 month after brachytherapy (Fig. 3). On the other hand, detection of PSA only or concurrently with hK2 mRNA in the peripheral blood during brachytherapy had predicted the kinetic pattern and values of PSA postoperatively (Figs. 3–5 and Table III). Because the detection rate of PSA, hK2 or PSA/hK2 mRNA increased significantly during brachytherapy (Table II and Fig. 3), it is clear that brachytherapy has an iatrogenic effect. The clinical significance of the differences in PSA kinetics and values is still not clear since our follow-up ranged from 3 to 5 years. Today, only one patient had biochemical failure and his PCR was positive for PSA mRNA during brachytherapy. Longer follow-up is necessary to assess the clinical worth of detection of prostate-specific transcripts in the peripheral blood during brachytherapy as well as their effects on disease-specific survival. In comparison with the intra-operative RT-PCR results during radical prostatectomy, some authors have found significant correlations with tumor grade and stage [13] while others failed to show any significant correlations [16].
Fig. 3. Posttreatment blood PSA levels in the different groups of patients. Mean PSA values (points) and their corresponding SE values (bars) were plotted over time in patients with positive versus negative PCR reactions to PSA before (A), during (B) and after brachytherapy (C), to hK2 during brachytherapy (D) and to simultaneous PSA and hK2 during brachytherapy (E). P values were calculated using two-way ANOVA as described under “Materials and Methods Section.” F: Normalized mean PSA to the corresponding initial mean PSA from (E) were plotted over time and best linear regressions were drawn to obtain the slope of PSA decline according to the equation: \(Y = -aX\), where \(Y = \log(PSA)\), \(a = \text{slope}, X = \text{time}\).
The significance of positive PCR reaction prior to brachytherapy is not clear and is likely not reliable. First, there was no significant correlation with the biochemical outcome (Fig. 3) and second, positive reactions did not persist 1 month after treatment (Table II). We therefore believe pretreatment PCR test is not accurate to predict extraprostatic disease or biochemical outcome.

**Fig. 4.** Postbrachytherapy monotherapy blood PSA levels in the different groups of patients. A: Mean PSA (points) and ±SE values (bars) were plotted over time in the Groups as categorized in “Table I.” Mean PSA (points) and ±SE values (bars) of Group I were plotted over time and separated according to positive and negative PCR results during brachytherapy to PSA (B), to hK2 (C), and to simultaneous PSA and hK2 (D). P values were calculated using two-way ANOVA as described under “Materials and Methods Section.” E: Normalized mean PSA to the corresponding initial mean PSA from (D) were plotted over time and best linear regressions were drawn to obtain the slope of PSA decline according to the equation: Y = -aX, where Y = Log[PSA], a = slope, X = time.
Several investigators in recent years have assessed various PSA kinetic parameters for their ability to predict biochemical recurrence and overall survival after the different treatment modalities for prostate cancer whether primary therapies (radiation and surgery) [36,37] or systemic therapies [38,39]. Therefore, obtaining quicker and lower PSA values is regarded as a surrogate for better treatment outcomes. The fact that PSA levels in the current report declined more rapidly (1.5- to 2.4-fold) in patients with negative PSA/hK2 PCR results during brachytherapy (Figs. 3F and 4E) and indeed reached a lower values at each time point following treatment (Fig. 5), could point towards a better outcome for these patients.

It is believed that the expression of prostate-specific genes in the peripheral blood reflects the presence of circulating epithelial prostate and/or prostate cancer cells. Since we have not isolated cells from the peripheral blood it is not certain whether the origin of the PSA and hK2 transcripts was obtained from circulating prostate cells or from circulating cell-free DNA and RNA [40–43]. Nevertheless, given that the rate of positive PCR reactions for PSA and hK2 in patients treated with ADT was significantly lower than in patients treated with brachytherapy only (Table IV) strongly suggests that the expression of these genes is prostate related. Converting the expression of prostate-specific genes in the peripheral blood could have a prognostic value as previously described by Sourla et al. [44]. Therefore, if future follow-up and studies will determine the clinical relevance of prostate-specific transcripts detection in the blood it would be conceivable to offer these patients androgen ablation therapy.

One of the limitations of our study is that we utilized a qualitative nested RT-PCR technique to detect PSA and hK2 mRNA. Albeit this, we believe our test is reliable. First, concurrent detection of PSA and hK2 increased specificity (Table III); the number of positive PCR reactions for concurrent PSA and hK2 before and after brachytherapy in our population with organ-confined disease decreased to 0% (Table II). Second, there was a significant correlation between posttreatment PSA kinetics and PCR results (Figs. 3 and 4). Obviously, ADT biased the results and affected the RT-PCR assay. ADT decreased blood PSA levels regardless of brachytherapy as well as affected the positive rates of RT-PCR reactions (Table IV). Therefore, the results obtained from the hormone naive patients (Group I) are the most accurate. The RT-PCR assay for PSA and PSA/hK2 in this group of patients gives the best sensitivity (0.91, 0.86) and specificity (0.75, 0.81) values (Table III).

**Fig. 5.** The portion of patients with PSA <0.5 ng/ml over time. The percentage of patients with PSA <0.5 ng/ml in all Groups (A) and in Group I (B) were plotted over time according to their PCR results to PSA and PSA/hK2 during brachytherapy. Differences between portions of patients with positive versus negative PCR results for PSA and PSA/hK2 were calculated, respectively, using $\chi^2$ analysis at each time point. *p < 0.00625 as Bonferroni correction was applied to 8 time points.

<table>
<thead>
<tr>
<th>TABLE III. Validity of RT-PCR Assays During Brachytherapy</th>
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<tr>
<td>All groups</td>
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<td>-------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
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<td>Specificity</td>
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<td>PPV</td>
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<td>NPV</td>
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All tests refer to RT-PCR reactions performed during brachytherapy. Positive and negative RT-PCR tests were analyzed against blood PSA values >0.5 ng./ml (presence of condition) and <0.5 ng/ml (absence of condition), at 3 years, respectively. PSA, prostate-specific antigen; hK2, human kallikrein 2; PPV, positive predictive value; NPV, negative predictive value.

The Prostate
CONCLUSIONS

Altogether, our short-term results suggest that testing the expression of PSA and hK2 mRNA in the peripheral blood during prostate brachytherapy may have a prognostic value. Longer follow-up and further investigations in the future are warranted to confirm the prognostic value of this RT-PCR assay in patients undergoing brachytherapy.

ACKNOWLEDGMENTS

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REFERENCES


TABLE IV. Androgen Deprivation Therapy Reduces Positive PCR Reactions During Brachytherapy

<table>
<thead>
<tr>
<th>Group</th>
<th>PSA PCR (+), no. (%)</th>
<th>hK2 PCR (+), no. (%)</th>
<th>PSA/hK2 PCR (+), no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n = 35)</td>
<td>16 (46)</td>
<td>14 (40)</td>
<td>9 (26)</td>
</tr>
<tr>
<td>II and III (n = 45)</td>
<td>9 (20)</td>
<td>4 (9)</td>
<td>3 (7)</td>
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<tr>
<td>P-value, 2-sided Pearson Chi-square</td>
<td>0.014</td>
<td>0.001</td>
<td>0.018</td>
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PSA, prostate-specific antigen; hK2, human kallikrein 2.


