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Clinical usefulness of RT-PCR detection of hematogenous prostate cancer spread

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Abstract Understaging is commonly associated with therapeutic failure of surgical intervention in apparently localized prostate cancers. Methods that specifically detect prostate cancer cells in the circulation may be able to identify metastatic cancers and thus aid in the selection of the most adequate therapy. The high sensitivity and specificity of the reverse transcriptase–polymerase chain reaction (RT-PCR) encouraged various groups to investigate the mRNA expression of prostate-specific markers in the peripheral blood of patients with prostate cancer. However, probably due to methodological differences, many contradictory results have been obtained with the markers studied so far: prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM). For this reason, clinical decisions should not be based yet on RT-PCR results. Future research and long-term follow-up on the patients may point out whether RT-PCR assays, following appropriate standardization, will have an additive value in prostate cancer staging and in prediction of tumor progression.

Key words Prostate cancer · Hematogenous spread · Reverse transcriptase–polymerase chain reaction (RT-PCR) · Prostate-specific antigen · Prostate-specific membrane antigen

Introduction

Adenocarcinoma of the prostate is currently the most commonly diagnosed cancer and the second leading cause of cancer mortality in men in Western countries [75]. Therapeutic approaches for prostate cancer depend on the stage of the malignancy. The surgical curability of clinically localized prostate cancer (T2 tumor) is based upon the complete excision of the prostate (i.e., radical prostatectomy). However, despite preoperative staging with techniques such as digital rectal examination (DRE), endorectal magnetic resonance imaging (MRI), computed tomography (CT) scan, bone scan and serum prostate-specific antigen (PSA) level, it remains difficult to discriminate those patients who have locally confined tumors from those who have extraprostatic prostate cancer (pT3 tumors) and lymph node metastases identified at lymphadenectomy (pN+ tumors) [7, 23, 28, 47, 54, 65, 76, 79, 92]. Approximately 50% of patients with clinically localized prostate cancer will relapse with locally recurrent or metastatic disease after removal of the primary tumor. *The question arises whether micrometastases, already present at the time of surgery and undetectable by conventional pathological methods, could have been the cause of these therapeutic failures.* Therefore, establishing clear clinical correlations could eliminate a significant number of prostate cancer patients from unnecessary aggressive treatment, potentially increase the cure rate in patients who undergo radical prostatectomy and predict treatment failure after potentially curative management.

The process of metastasis is a dynamic process that does not occur randomly but is the result of a complicated series of tumor-host interactions (invasion, angiogenesis, intravasation, circulation, arrest, extravasation and growth), as shown in Fig. 1 [64]. From rapidly growing prostate tumors, thousands of tumor cells can end up in the circulation every day. After entering the circulation lymphatically or hematogenously, prostate tumor cells spread to distant sites in the body [4, 64]. Because of the hostile environment in the circulation, only a small percentage of the circulating tumor cells is believed to survive and to proliferate in secondary sites. Metastasis can therefore be regarded as an inefficient process [1, 64]. Metastases to the lymph nodes occur most frequently, followed by the bones, lungs, bladder

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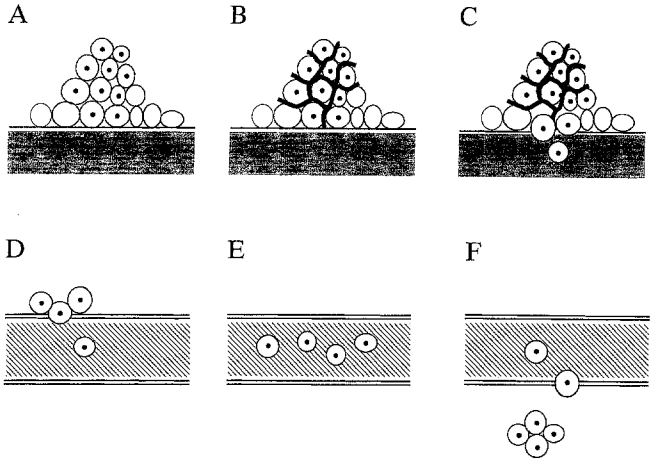

Fig. 1 The metastatic cascade: primary tumor formation (**A**), angiogenesis (**B**), local invasion (**C**), intravasation (**D**), circulation of invasive cells (**E**), extravasation and growth (**F**)

and liver and to a lesser extent other tissues [80]. As stated above, the penetration of cancer cells into the bloodstream is *one* important event in the metastatic cascade. At present, for patients with metastatic disease, the long-term prognosis is predictably poor [30] and treatment is aimed at slowing down the progression of the metastatic process. *Therefore, if normal prostatic cells do not circulate in peripheral blood, a sensitive method of detecting circulating prostate tumor cells in patients with prostate cancer may be an indicator of the extent of a tumor and of its metastatic potential.*

Several methods have been developed to detect circulating prostate tumor cells in peripheral blood of patients with prostate cancer. Hamdy et al. [37] performed an analytical flow cytometry study, to detect PSA-positive circulating tumor cells in patients with newly diagnosed, untreated prostate cancer. Although there was some doubt about the prostatic origin of the PSA-positive cells [26, 37], their presence was related to the presence of metastatic disease, confirmed by bone scans, and was a more reliable predictor of disseminated disease than total serum PSA levels. Recently, due to its high assay sensitivity and specificity, reverse transcriptase–polymerase chain reaction (RT-PCR) has been used to investigate the presence of circulating tumor cells of different kinds of neoplasms, including prostate cancer [31]. RT-PCR is a gene expression assay by which mRNA levels in a cell can be detected. If the mRNA is specific for a certain type of cell, RT-PCR can identify the presence of one such cell, either isolated or mixed with thousands or even millions of other cells. *Therefore prostate markers, such as PSA and prostate-specific membrane antigen (PSM), are promising candidates for evaluating the utility of RT-PCR in detecting circulating prostate cells.*

This review will summarize the results of the detection of circulating prostate tumor cells in the peripheral blood of patients with prostate cancer by RT-PCR for PSA and PSM and discuss the possible usefulness of this method in staging for prostate cancer.

- The question arises whether micrometastases, already present at the time of surgery and undetectable by conventional pathological methods, could have been the cause of therapeutic failures.
- If normal prostatic cells do not circulate in peripheral blood, a sensitive method of detecting circulating prostate tumor cells in patients with prostate cancer may be an indicator of the extent of a tumor and of its metastatic potential.
- Prostate markers, such as PSA and PSM, are promising candidates for evaluating the utility of RT-PCR in detecting circulating prostate cells.

PSA and PSM as prostate markers and their role in staging clinically localized prostate cancer

PSA is a 34 kDa, seminal serine protease that belongs to a subgroup of kallikreins, with high similarity to human glandular kallikrein (hGK-1) [39]. In semen, it is responsible for liquefaction of the seminal coagulum following ejaculation. *PSA is expressed under androgen control by the ductal epithelial cells of the prostate and secreted into the lumen of the gland. PSA protein can be detected in male serum as a result of leakage into the blood [3, 22].* The amount of PSA in the circulation increases with the volume of the tumor and with advancing degree of dedifferentiation, which contributes to the usefulness and informative value of total serum PSA measurements in early diagnosis, staging and disease progression [3, 5, 22, 54, 56, 76, 88]. However, the measurement of serum PSA level also has its limitations. It is not specific to cancer and distinguishing benign prostatic hyperplasia (BPH) from localized prostate cancer on the basis of total serum PSA concentrations is difficult, as the observed ranges of serum PSA concentrations in patients with BPH and prostate cancer overlap. However, by combining several staging and grading techniques such as serum PSA and the Gleason score, or adjusting the serum PSA measurements for variables such as patient age, prostate volume and cancer volume, the predictive power of serum PSA for the final pathological stage can be enhanced [3, 5, 54, 76].

Recently an additional prostate biomarker, the type II transmembrane glycoprotein PSM, has been described [41, 43]. It appears as a predominant 120 kDa and minor 200 kDa band in prostate tissue and seminal plasma. *It is expressed within the epithelial cells of normal prostate, BPH and, to a greater extent, in malignant prostatic tissues [41, 44, 91]. In contrast to PSA, PSM expression is down-regulated by androgens [44].* Studies on the presence of PSM protein in the serum of both normal individuals and prostate cancer patients gave some conflicting results. Although one group [91] could not detect specific PSM expression in serum from prostate carcinoma patients, other studies demonstrated PSM in serum in significant amounts by either an immunoassay or Western blot

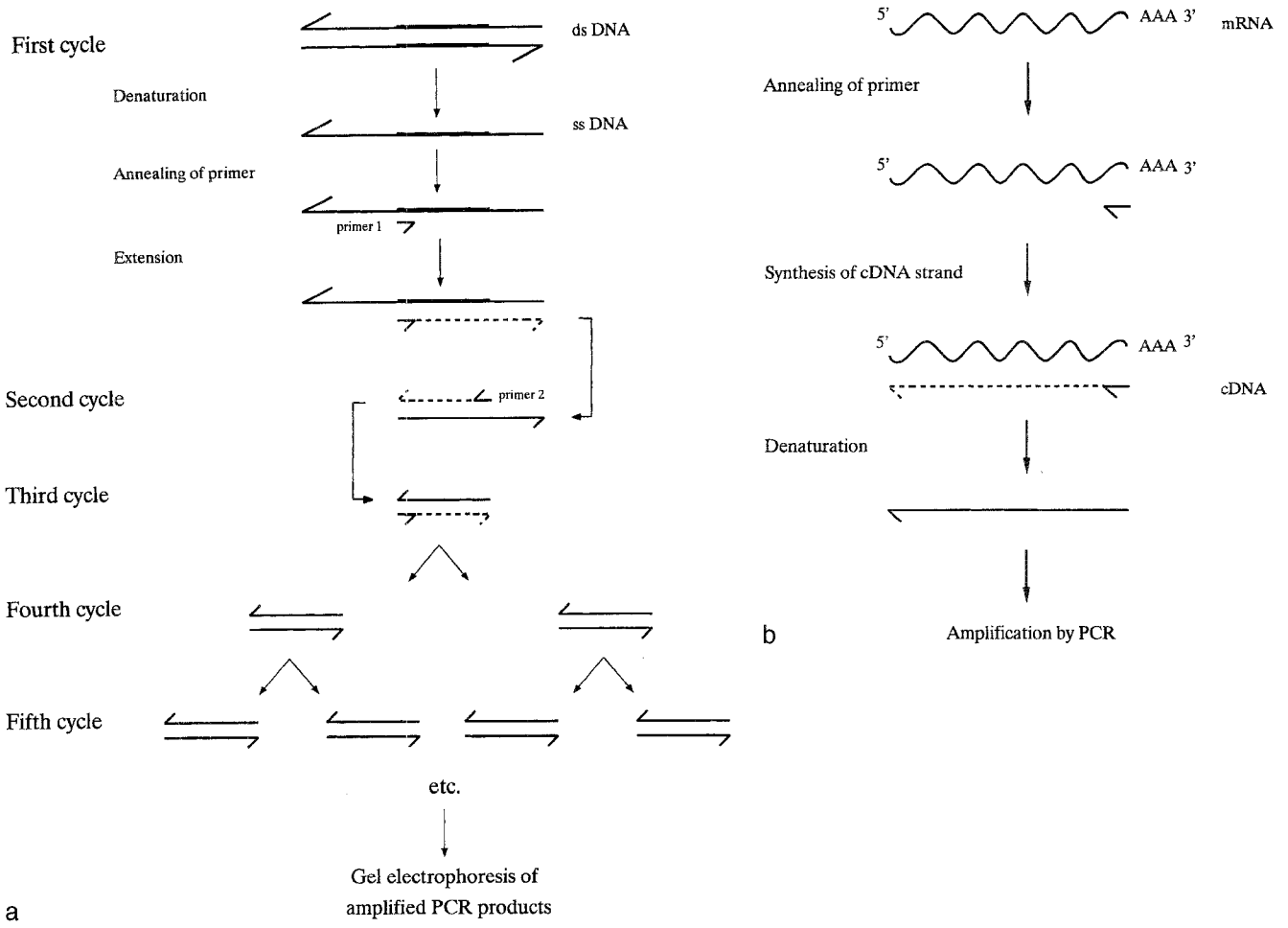

Fig. 2 a Selective amplification of a DNA sequence (indicated by a *black bar*). The double-stranded DNA is denatured to single strands and annealed to one of the oligonucleotide primers, which are orientated so that when it is extended by the action of DNA *Taq* polymerase the newly synthesized strands will overlap. The process of denaturation, annealing and extension is repeated many times, resulting in an exponential accumulation of the specific strands **b** Reverse transcription of mRNA to cDNA by reverse transcriptase

[41, 66–68, 78]. Serum PSM levels in patients with prostate cancer were significantly higher than those in normal individuals. Elevated levels predicted a state of clinical progression or resistance to treatment in most cases, usually associated with the presence of metastases [41, 66, 67]. These results indicate that PSM is expressed in the human prostate and furthermore that it may be a marker for aggressive clonal populations of prostate cancer cells due to its increased expression in hormone-resistant tumors [41, 44, 66, 67]. However, at present PSA provides the best information indicating clinical progression in the presence of clinically localized prostate cancer. It is possible that PSM is applicable to clinical situations different from those assessed by PSA. Further investigations are awaited to determine whether PSM is a useful marker for prostate cancer.

- PSA is expressed under androgen control by the ductal epithelial cells of the prostate and secreted into the lumen of the gland. PSA protein can be detected in male serum as a result of PSA leakage into the blood [3, 22].
- PSM is expressed within the epithelial cells of normal prostate, BPH and, to a greater extent, in malignant prostatic tissues [41, 44, 91]. In contrast to PSA, PSM expression is down-regulated by androgens [44].

RT-PCR method

The polymerase chain reaction (PCR) is a relatively simple, but very sensitive and highly specific technique for amplifying small amounts of DNA. The technique involves the in vitro enzymatic synthesis of millions of copies of a specific DNA fragment and is based on repeated cycles of DNA denaturation, annealing and extension of two oligonucleotide primers (short DNA sequences of about 20–25 nucleotides) that flank the target region (Fig. 2A) [9, 24, 33]. After denaturation of the DNA, each primer hybridizes to one of the separated strands, such that extension from each 3′ hydroxyl end is directed towards the other. When the primers are subsequently extended by a thermostable DNA polymerase, the newly synthesized strands will overlap the binding site of the opposite primer. As the process of denaturation, annealing and extension is continued, the primers repeatedly bind to

both the original DNA template and complementary sites in the newly synthesized strands, resulting in an exponential accumulation of a specific fragment whose ends are determined by the 5′ ends of the primers. Although the PCR assay is a very sensitive assay, an additional nested PCR (reamplification of an aliquot of the PCR product using a different set of primers that are complementary to the sequence of the first product), Southern blotting (hybridization of the PCR products with a specific radioactive DNA fragment), the use of direct incorporation of radioactive labelled primers followed by autoradiography or digoxigenin detection (chemiluminescent enhancement of PCR products with the use of an antibody) may increase the signal.

An important variation on the conventional PCR protocol is RNA-PCR or RT-PCR [33]. *RT-PCR is similar to DNA PCR except that PCR amplification is preceded by reverse transcription of RNA into complementary DNA (cDNA).* The entire RT-PCR assay consists of four steps: (1) total RNA extraction of the isolated nucleated blood cells or tissue; (2) the synthesis of cDNA from total RNA by viral reverse transcriptases utilizing random hexamers, a gene-specific antisense primer or an oligo-dT primer (Fig. 2B); (3) the amplification of the target sequence with gene-specific sense and antisense primers, as in the standard PCR reaction and (4) analysis of the PCR products by agarose gel electrophoresis. Protocols for RNA isolation and cDNA synthesis prior to PCR amplification have varied greatly with respect to the details of cDNA priming and the initial sample preparation, but all follow the same scheme. The RT-PCR assay can be quantified by using an internal control fragment which possesses a small deletion in the amplified portion of the molecule [77, 87]. This standard fragment can be incorporated into the PCR reaction but would yield a product of different length and can be distinguished easily by polyacrylamide gel electrophoresis from the original fragment.

Two general approaches have been studied utilizing the RT-PCR assay. First, neoplastic cells could be recognized in the blood as a means of verifying hematogenous cancer cell spread. Secondly, neoplastic cells could be detected in the regional lymph nodes or in bone marrow as a means of verifying the presence of regional and distant cancer cell spread. These studies suggest that by RT-PCR it is possible to detect a single cell from hepatocellular carcinoma [50, 60], breast cancer [97], melanoma [83] or neuroblastoma [61] in peripheral mononuclear blood cell dilutions up to $1{:}10^6$.

Considerations in applying the RT-PCR assay

In practice, care must be taken when applying the RT-PCR assay to avoid false positive results, by careful choice and handling of samples and controls. Contamination of genomic DNA, exogenous RNA or cDNA in a sample can be amplified to a detectable level. Obviously, the fewer molecules one is trying to detect, the more one should guard against this possibility. At present, contamination forms a serious limitation in the use of (RT) PCR for clinical testing and also for some research applications. To avoid false positives with RT-PCR, physical transfer of RNA and cDNA between different samples and also between positive and negative experimental controls should be prevented. Kwok and Higuchi [55] have published a list of guidelines for (RT) PCR which dramatically reduced false positive rates in their laboratory. This list includes, among others, autoclaving of solutions, aliquoting of reagents, use of tips with barriers preventing contamination and avoiding splashes.

Besides these practical precautions, positive and negative controls should be chosen and handled carefully. The reagent controls should contain all the necessary components for RT-PCR, except template RNA. In many cases, the PCR primers can be designed to specifically amplify the cDNA sequences and not the genomic DNA by choosing intron spanning primers. However, when the exon-intron structure of the gene of origin is unknown or an intronless gene is studied, it is not possible to distinguish genomic DNA from amplified cDNA sequences. A DNase pretreatment under specified conditions [35] may resolve this problem. An alternative way to eliminate false positive signals caused by DNA contamination in a RT-PCR reaction is described by Dougherty et al. [16]. They developed a restriction endonuclease digestion assay in which a restriction enzyme, selected to cut the DNA between, but not within, the primer binding sites, was used to digest reaction mixtures after reverse transcription but before PCR amplification. Because restriction enzymes generally react only with specific double-strand sequences, contaminating DNA was made inactive while reverse transcribed single-strand cDNA was amplified. Several approaches to minimize another frequently occurring problem – the potential to carry over previously amplified product – have been developed. These are all based on interfering with the ability of the amplified product to serve as a template. One such strategy utilizes a 3′-terminal ribose residue primer [93]. Extension of the primer by *Taq* DNA polymerase generates a cleavable ribonucleotide linkage within the amplified product. Cleavage of the primer by base or ribonuclease treatment prevents reamplification when the product is carried over to another sample. A last consideration is the risk of misinterpreting negative results, as described by Melo et al. [62]. These authors highlight the difference between *possibility* and *reproducibility* of detection of PCR products. Awareness of this difference is particularly important when a single negative result is relied on to demonstrate the lack of gene expression.

If all the above-mentioned precautions are taken into consideration, *the current tendency to exploit the power of (RT) PCR amplification strategies to their limit is valid so long as technical artifacts can be avoided.*

- The polymerase chain reaction (PCR) is a relatively simple, but very sensitive and highly specific technique for amplifying small amounts of DNA.

- An important variation on the conventional PCR protocol is RNA-PCR or RT-PCR [33]. RT-PCR is similar to DNA PCR except that PCR amplification is preceded by reverse transcription of RNA into complementary DNA (cDNA).
- The current tendency to exploit the power of (RT) PCR amplification strategies to their limit is valid so long as technical artifacts can be avoided.

Clinical significance of the detection of PSA- and PSM-positive circulating tumor cells by means of RT-PCR

Prostate cancer cells can be identified by RT-PCR of mRNA encoding for PSA or PSM. In theory, and with adequate numbers of cases, combining the results of an RT-PCR assay with other clinical parameters may improve case selection for local treatment approaches, identify progression early in the clinical course and allow more precise definition of disease-free status. To this end, different groups have explored the usefulness of RT-PCR targeted at the PSA and PSM genes as a detection method for circulating prostate tumor cells in peripheral blood of prostate cancer patients at different stages. The results of these studies are summarized in Table 1. All tests were designed to be free of interference by genomic DNA and had an assay sensitivity of at least 1 lymph node carcinoma of the prostate (LNCaP) cell per 10^6 leukocytes. This detection limit should be sufficient considering that a standard buffy coat contains about 10^6 white blood cells, depending on the volume used.

In 1992, Moreno et al. [63] first described the detection of PSA-positive cells in the peripheral blood using a RT-PCR technique. They addressed the question whether patients with advanced prostate cancer (stage D0–3) have detectable hematogenous prostate tumor cells, using the RT-PCR assay with PSA-specific primers. Of the 12 patients, four were found positive for PSA mRNA expression: two with stage D1, one stage D2 and one stage D3. All patients with non-metastatic disease, i.e., stage D0, were negative. Some explanations were given for the fact that not all patients with metastatic disease had detectable circulating PSA-positive cells. First, the entire Ficoll layer was used for RNA isolation, while prostate cancer cells are preferentially found in the buffy coat. Second, circulating clonal populations of prostate cancer cells may be poorly differentiated, thereby expressing low levels of PSA. Another study also found that only a proportion (50%) of patients with metastatic prostate cancer were RT-PCR positive for PSA [46]. Most of the cancer patients in this study received endocrine treatment that may have suppressed PSA expression, thereby explaining the high percentage of negative results [46].

A positive RT-PCR result for patients with advanced prostate cancer does not provide extra information regarding choice of treatment, because it is already known that these patients have metastases. However, this group of patients should serve as an important positive control population. *In contrast, the evaluation of the blood of patients with localized prostate cancer is far more interesting, because appropriate management of this population is complicated by both the imperfect clinical staging techniques, as stated earlier, and the variable natural history of prostate cancer.* Therefore some groups examined a small population of patients with clinically localized prostate cancer. Seiden et al. [82] reported that by using a nested RT-PCR assay for PSA, circulating prostate tumor cells could be detected in 8% of patients with untreated clinically localized prostate cancer and in 31% of patients with metastatic disease. Of the latter, 91% (10/11) had hormone-refractory disease. On the contrary, only one out of eight patients with hormone-sensitive metastatic disease was PSA-positive. The RT-PCR assay of PSA mRNA in the blood of men with elevated serum PSA protein levels but no documented malignancy was uniformly negative, which suggests high specificity of the assay used. Ghossein et al. [32] evaluated peripheral blood specimens of patients with prostate cancer in clinical stages varying from T1c to M+ for the presence of PSA mRNA. The frequency of positivity in this study increased with tumor stage, with a maximum of 34% of patients with distant metastases, again indicating a low clinical sensitivity. Interestingly, among the patients with hormone-sensitive metastatic

Table 1 Summary of the results of the blood-based PSA and PSM RT-PCR studies

Authors	Marker	Negative controls (%) positive	≤pT2 N0M0 (% positive)	>pT2 N0M0 (% positive)	Metastatic disease (% positive)
Cama et al. [6][a]	PSA	0/65 (0)	6/48 (13)	21/32 (66)	16/20 (80)
	PSM	0/65 (0)	10/48 (21)	9/32 (28)	10/20 (50)
Ghossein et al. [32]	PSA	0/27 (0)	1/17 (6)	5/14 (36)	26/76 (34)
Israeli et al. [45]	PSA	1/40 (3)	0/18 (0)	1/15 (7)	6/24 (25)
	PSM	2/40 (5)	13/18 (72)	9/15 (60)	16/24 (67)
Jaakkola et al. [46]	PSA	0/18 (0)	0/2 (0)	0/3 (0)	9/18 (50)
Loric et al. [59]	PSA	0/53 (0)	1/17 (6)	2/10 (20)	17/33 (51)
	PSM	0/53 (0)	6/17 (35)	6/10 (60)	28/33 (85)
Moreno et al. [63]	PSA	0/17 (0)			4/12 (33)
Seiden et al. [82]	PSA	0/14 (0)	5/65 (8)[b]		11/35 (31)
Sokoloff et al. [85]	PSA	1/19 (5)	30/51 (59)	13/18 (72)	19/33 (88)
	PSM	1/19 (5)	8/51 (16)	4/18 (22)	13/33 (39)

[a] Other reports have been published by this group [51–53, 73, 74]

[b] Clinically localized prostate cancer, not pathologically confirmed

disease, one third were PSA RT-PCR positive while all had normal or undetectable PSA serum levels after hormone therapy. According to the authors, this suggests that hematogenous spread of PSA-positive cells may still be occurring in these patients, despite seemingly successful therapy.

Application of the RT-PCR technique to preoperative prostate cancer staging was suggested by Katz et al. [51]. They developed a digoxigenin-enhanced detection method for PSA RT-PCR products. With this modified method, 39% of patients with clinically localized prostate cancer and 78% of those with untreated metastatic prostate cancer were PSA mRNA-positive, which is a higher percentage than found by the previously mentioned studies [32, 46, 63]. *Control subjects, patients without prostate cancer such as women, young men and men with BPH, were consistently negative.* PSA positivity of the localized cancers correlated with final pathological stage (positive margins, capsular penetration and positive seminal vesicle). Used as an independent factor to predict tumor extension beyond the prostate gland, RT-PCR for PSA had a positive predictive value of 80%, which was higher than CT scan and MRI. From these results the authors suggested that the blood-based digoxigenin-enhanced RT-PCR assay for PSA may be of value in correctly staging prostatectomy patients before surgery. Additional studies using an extended patient pool confirmed these findings [52, 53, 73, 74]. According to the most recent update [53], at which time 160 radical prostatectomy patient had been screened, clinically localized prostate cancer patients with a positive PSA mRNA RT-PCR result are 16.7 times more likely to have extraprostatic disease than those with a negative reaction. With a mean follow-up period of 11.9 months (range 1–31 months), patients who were RT-PCR positive prior to radical prostatectomy were 4.1 times more likely to have biochemical recurrence, defined by a rise in PSA serum equal to or above 0.2 ng/ml [53]. In conclusion, Katz et al. [51–53] and Olsson et al. [73] showed that the diagnostic properties of the RT-PCR assay had a higher positive and negative predictive value than other staging modalities tested, including DRE, imaging, serum PSA level and Gleason grade. Examining the ability of the RT-PCR assay to stage accurately over varying PSA ranges, it was found that RT-PCR for PSA in the category of patients with high serum PSA levels (>10 ng/ml) discriminated between potentially curable candidates and those with established extraprostatic disease [53, 73].

Comparative studies on the expression of PSM have been reported. A nested RT-PCR assay with primers derived from the PSA and PSM gene, was used by Israeli et al. [45] to detect hematogenous spread of prostate cells in patients with different stages of prostate cancer. Primer specificity was confirmed by Southern blotting of the RT-PCR products. Although the PSM and PSA primers yielded similar assay sensitivities in LNCaP dilution curves, PSM primers detected prostate tumor cells in 67% of the total group of prostate cancer patients, whereas PSA primers detected 12%. In contrast to the published data of Katz et al. [51–53] and Seiden et al. [82], the PSA RT-PCR method was unable accurately to detect circulating PSA-positive cells in patients with pathologically organ-confined prostate cancer or metastasized disease in this study. In patients with documented metastatic prostate cancer who were receiving anti-androgen treatment, 67% were PSM-positive and 25% PSA-positive. In a group of seven patients with hormone-refractory prostate cancer, six, who all died within 2–6 months, were positive for both PSM and PSA mRNA. The one patient who tested negatively was still alive 15 months after the assay. Because none of the 17 patients with hormone-sensitive stage D disease were PSA-positive, against 10 of 17 for PSM, the authors suggested that PSA-positivity may serve as a predictor of early mortality. It should be noted that 2.5% of the negative controls for PSA and 10% for PSM were positive, meaning that the blood samples from the negative control group had a detectable amount of PSA- or PSM-positive cells. Half of the PSM "false" positives represented patients with elevated serum PSA values and an enlarged prostate. The serum levels in these patients continued to rise and, at a later date, prostate biopsy revealed prostate cancer, suggesting that PSM expression may predict the development of cancer in patients without clinically apparent prostate cancer [45].

A second study comparing PSM and PSA mRNA expression was done by Loric et al. [59]. They performed nested RT-PCR based on PSM and PSA, using different PSA- and PSM-specific primers from those used by Israeli et al. [45]. In patients with metastatic disease, who had all been treated by androgen deprivation, 85% were PSM-positive and 51% PSA-positive. All patients with hormone-refractory metastatic disease were PSM-positive. Although analysis of a larger blood sample (20 ml) increased the percentage PSA-positives, it still failed to detect 30% of the patients with metastatic prostate cancer. Among the patients with pathologically confirmed localized prostate cancer, 44% were PSM-positive and 11% PSA-positive. Taken together, these results support the previous finding by Israeli et al. [45] that the use of PSM instead of PSA primers improved the clinical sensitivity of the RT-PCR and that PSM-expressing cells in blood may predict the development of cancer in patients without metastatic prostate cancer.

Cama et al. [6] repeated the initial experiments done by Katz et al. [51, 52] using the same patients' samples to evaluate whether the use of a PCR primer set designed to amplify the PSM gene was as useful in distinguishing patients with early cancer spread as the previously developed primers for PSA. Of the patients with untreated metastatic disease, 80% were PSA-positive and 50% PSM-positive. Among the patients with clinically localized prostate cancer, 34% were PSA-positive and 24% PSM-positive. In contrast to the report by Israeli et al. [45], Cama et al. [6] identified a statistically significant stronger correlation of tumor invasion with the PSA results (70% clinical sensitivity) compared with the PSM

results (33% clinical sensitivity). This may be explained by the fact that none of the metastatic prostate cancer patients had undergone prior therapy, while PSM is known to be down-regulated by androgens and thus derepressed or up-regulated by anti-androgens or androgen deprivation.

A recent study by Sokoloff et al. [85] supported the results of Cama et al. [6]. They applied a quantitative autoradiography-enhanced RT-PCR assay to measure the degree of tumor burden in the circulation of patients with localized and hormone-refractory metastatic prostate cancer. In all different pathological stages a higher percentage of patients were PSA-positive than were PSM-positive (Table 1). One patient of the control group was both PSA- and PSM-positive, resulting in a percentage of false positives of 5% for this assay. Neither a positive PSA or PSM signal nor the quantity of these signals could reliably predict pathological stage. Furthermore, it was demonstrated that there was no statistically significant relationship between RT-PCR results and preoperative serum PSA, Gleason score, apical involvement and capsular penetration [85].

Except for the last study [85], in none of the PSM-based RT-PCR studies performed was it known whether the PSM primers used were intron spanning. Contamination of the RNA samples by genomic DNA therefore had to be considered as a potential problem in each of these studies; however, negative controls suggest that at least in some this was not the case. Heston [40], having sequenced 90% of the PSM gene, confirmed that the primers used in the different PSM studies are appropriate for use in RT-PCR, as the amplified cDNA fragment does indeed cross an exon-intron junction.

RT-PCR is a powerful method that is able to detect PSA- and PSM-positive cells in the circulation of patients with prostate cancer. *Unfortunately, separate studies have given different results about the predictive value of PSA or PSM RT-PCR results. Discrepancies between studies may partially be the result of the use of different RT-PCR protocols.* Because different amplification cycle numbers, different reaction conditions and different PCR primer sets were used for both PSA and PSM (Table 2), it is difficult or even impossible to compare the results obtained in the various studies [48]. Henke et al. [38] tested several combinations of published primers, including the primers used by Katz et al. [51], Moreno et al. [63] and Israeli et al. [45], in a nested RT-PCR. This resulted in different assay sensitivities, indicating that primer selection is decisive for the sensitivity of PCR. Therefore, it should be emphasized that the establishment of RT-PCR assays requires a significant effort in standardization of the protocol and quality control management, which would result in a more meaningful reporting of these values. *Apart from this, evaluation of the clinical significance of detection of hematogenous prostate cells by means of RT-PCR will require critical long-term follow-up data of a large number of patients.* At the moment, clinical decisions on the basis of RT-PCR results can not be made.

- The evaluation of the blood of patients with localized prostate cancer is far more interesting than that of patients with metastatic disease, because appropriate management of this population is complicated by both the imperfect clinical staging techniques, as stated earlier, and the variable natural history of prostate cancer.
- Control subjects, patients without prostate cancer such as women, young men and men with BPH, were consistently negative.
- Unfortunately, separate studies have given different results about the predictive value of PSA or PSM RT-PCR results.
- Discrepancies between studies may partly be the result of the use of different RT-PCR protocols.
- Besides standardization of the protocol, evaluation of the clinical significance of detection of hematogenous prostate cells by means of RT-PCR will require critical long-term follow-up data of a large number of patients.

Discussion

What do we expect from new and promising marker technology? First, it should improve the differentiation

Table 2 Differences in methodology

Authors	RT-PCR method	Marker	Amplification cycles	Primers	
				Position	Length (bp)
Cama et al. [6][a]	Enhanced[b]	PSA	33	Exon 3/5	710
		PSM			410
Ghossein et al. [32]	Enhanced[c]	PSA	28	Exon 3/4	217
Israeli et al. [45]	Nested	PSA	50 (2 × 25)	Exon 4/5[e]	355
		PSM			234
Jaakkola et al. [46]	Nested	PSA	60 (2 × 30)	Exon 4/5[e]	194
Loric et al. [59]	Nested	PSA	50 (2 × 25)	Exon 3/5[e]	261
		PSM			196
Moreno et al. [63]	Standard	PSA	28	Expm 3/4	214
Seiden et al. [82]	Nested	PSA	70 (2 × 35)	Exon 2/3[e]	64
Sokoloft et al. [85]	Enhanced[d]	PSA	28		195
		PSM			308

[a] Other reports have been published by this group, all using the same RT-PCR protocol [51–53, 73, 74]
[b] Digoxigenin-enhanced
[c] Enhanced by a radioactive DNA probe
[d] Enhanced by direct incorporation of radioactive primers
[e] Inner primers

between normal men, men with BPH and men with prostate cancer. Furthermore the marker should differentiate between clinically relevant and non-aggressive small lesions; contribute to staging of local organ-confined and non-organ-confined prostate cancer (better than DRE, TRUS, Gleason score and serum PSA); predict metastatic potential and aggressiveness of clinically localized lesions; predict endocrine responsiveness and independence; and, finally, function as a surrogate marker for the prediction of progression under therapy. Some of the data currently available suggest that RT-PCR detection of PSA and PSM mRNA may fulfil some of these requirements but *the evaluation of the clinical correlations is difficult due to the lack of standardization and the incompleteness of patient follow-up data in most studies.*

PSA has already been detected by RT-PCR analysis in bone marrow and lymph nodes of patients with organ-confined prostate cancer and a higher percentage of patients with metastatic disease [12, 13, 19, 94, 95]. More importantly, in a retrospective analysis of formalin-fixed paraffin-embedded pelvic lymph node material from patients who had undergone radical prostatectomy, Edelstein et al. [19] showed that 88% of the patients who were RT-PCR positive for PSA mRNA manifested rising serum PSA values within 5 years postoperatively, in contrast to only 30% of the RT-PCR negative patients. In other words, RT-PCR positivity for PSA mRNA was significantly correlated with early relapse.

In this review, the results of PSA and PSM RT-PCR analysis on the peripheral blood of patients with prostate cancer are summarized. *Most reports conclude that the RT-PCR assay has a low clinical sensitivity (percentage of patients with metastatic disease who are RT-PCR positive).* The studies of Olsson and coworkers showed that RT-PCR positivity for PSA correlated with final pathological stage and had a predictive value for surgical failure following radical prostatectomy [53]. These correlations could not be shown by others. Studies on PSM expression were not unambiguous either. Data presented at the Annual meeting of the American Urological Association 1996 and 1997 emphasized the contradictory results published [18, 21, 29, 42, 71, 86, 89, 96]. In conclusion, despite the fact that most of the RT-PCR studies for PSA mRNA had comparable assay sensitivity and patient groups, the results varied widely with respect to clinical sensitivity (metastasis detection) and correlation with clinical findings. To provide relevant information for clinical urologists, appropriate follow-up data will be needed. To date only Katz et al. [53] have reported on studies with adequate follow-up.

A number of factors may have caused the discrepancies between the various studies as noted above. PSA and PSM expression may be heterogeneous. Furthermore, prostate tumor cells may enter the circulation intermittently. This may lead to sampling errors, as only a few milliliters of blood are analyzed. Even if the tumor cells were shedded into the circulation continuously, a routine RT-PCR analysis has a probability of 63% for the detection of tumor cells at high dilutions [48]. This can be improved to 99.3% either by using a larger RNA sample or by repetitive testing of identical samples. However, it is not likely that the above-mentioned phenomenon alone could account for the discrepancies, since some studies did have a high clinical sensitivity.

An RT-PCR test may specifically detect low concentrations of PSA and PSM mRNA in non-prostatic cells, thereby decreasing the clinical sensitivity. PSA was originally thought to be prostate tissue-specific. However, Diamandis et al. [15] demonstrated that 30% of breast tumors contain significant amounts of PSA in their cytosol and it appears to be a favorable prognostic indicator in breast cancer. Recent studies have shown that also normal breast, biological fluids such as breast milk [98], normal salivary gland, normal pancreas [20], normal endometrium [10] and some lung [58], ovarian, liver, kidney, adrenal, colon and parotid tumors [57] contain immunoreactive PSA. PSM has also been detected in tissues other than the prostate. At very low levels, it was detectable in small intestine, salivary gland and the human brain [44, 91], although another study [68] could not confirm its expression in the brain. These results may not be very surprising, considering the fact that prior reports with assay-sensitive PCR methods also found promiscuous transcription of other so-called tissue-specific genes [8, 81].

Since the expression of PSA or PSM in non-prostatic tissue is commonly very low, it is not expected to interfere with the clinical usefulness of PSA- or PSM-based detection and monitoring prostate cancer [34]. Indeed, except for two controls in the study of Israeli et al. [45] and one in the study of Sokoloff et al. [85] that were PSA- and/or PSM-positive for an unknown reason, controls such as females, young males, men with BPH and men with other (urological) malignancies, are consistently negative in the blood-based RT-PCR. *Thus, in practice, PSA and PSM mRNA detection in the RT-PCR assay seem to be specific for patients with prostate cancer.* Nevertheless, the search for alternative markers with a more prostate-restricted expression is still continued. Prostate-specific transglutaminase (hTGp), a member of the transglutaminase family of calcium-dependent enzymes that catalyze the post-translational crosslinking of proteins, may represent such a marker [17].

Besides properties of the tumor itself, technical factors may contribute to the differences in the results obtained. As emphasized above, assay standardization is needed, since no two testing sites are using the same PCR procedures, especially with regard to primer selection. When no study is reproducing the exact technical specifications of any previous study it is difficult to compare the results in terms of whether groups are "reproducing" each other's data. Currently attempts are being made to develop a standardized, reproducible assay. The use of a recombinant PSA plasmid as an exogenous internal control gives information about the PCR assay performance, and reduces the number of "false negatives" [11]. Takahashi et al. [90] developed a

rapid colorimetric enzyme-linked immunosorbent assay (ELISA) as a first step toward standardizing molecular staging assays. RT-PCR procedures are continuously being modified with the aim of lowering the detection limit. But what level of sensitivity is actually necessary for this assay? A standard blood aliquot provides about 10^6 white blood cells. In this case, a detection limit of 1 in 10^6–10^7 would be satisfactory. However, when analyzing, for example, bone marrow samples, the detection limit has to be lower, because a bone marrow sample contains more cells. Furthermore, the method currently used to evaluate the assay sensitivity, LNCaP dilution curves, gives only an rough estimation, because LNCaP cells vary in their PSA and PSM mRNA production between laboratories and under different culture conditions. This is not to say that there is no lower bound to the detection limit desired. Smith et al. [84] detected low levels of PSA mRNA in all normal blood and bone marrow samples tested and several non-prostatic cell lines using a nested RT-PCR assay. When fewer cycles or just a standard primary PCR reaction followed by Southern blotting were performed, a lower percentage or even none of the non-prostate cancer samples were positive, indicating the low expression levels in these samples. *PSA and PSM RT-PCR may ultimately be clinically useful, however, as with most medical testing the clinical specificity and sensitivity will not be absolute and will need to be balanced.*

What is the possible clinical significance of a positive RT-PCR assay result? RT-PCR is able to detect PSA- and PSM-expressing cells in the circulation of patients with prostate cancer, in dilutions up to $1{:}10^7$. Assuming that $\pm 0.01\%$ of the total of circulating tumor cells actually form metastases, a concentration of 2 prostate cells per milliliter would be sufficient for hematogenous tumor spread [64]. Using a 5-ml blood sample, this number of cells is detectable by RT-PCR. *However, a positive RT-PCR result only indicates the presence of circulating tumor cells in the peripheral blood and does not necessarily imply that metastasis will occur or has occurred.* Undoubtedly, finding circulating cells will not predict a good outcome for the patient, but considering the complicated sequence of events necessary for tumor dissemination, the finding of circulating tumor cell clones may have little clinical relevance.

Despite the fact that the present data are not conclusive, they do have value because they contribute to the understanding of the mechanisms associated with prostate cancer metastasis. The studies demonstrated that a proportion of the patients with pathologically organ-confined prostate cancer or local extension of the tumor, but with negative lymph node involvement and bone scans, are found to be RT-PCR positive for PSA and/or PSM. *Although the biological significance of these prostate cells in the circulation is not known, these patients may represent a subset of patients who will relapse with metastatic disease or suffer tumor recurrence after an initial complete response to therapy.* Identifying this group at an early stage will limit the number of unnecessary operations. Besides staging prostate cancer, a second application of the RT-PCR assay could be to monitor disease progression after radical prostatectomy. However, a positive RT-PCR result postoperatively can be explained not only by surgical failure but also by intraoperative hematogenous dissemination of prostate cells. Although there is some evidence that hematogenous dissemination during radical prostatectomy occurs [25, 49, 69, 70], the clinical significance remains uncertain [2, 14, 36, 72]. A preliminary study by Fair et al. [27] showed that the use of neoadjuvant hormone treatment before radical prostatectomy substantially reduced the postoperative PSM RT-PCR positivity and thereby the number of potentially "cured" patients.

For now, the central question of whether RT-PCR positivity reliably predicts tumor stage and relapse remains unanswered. To be useful for clinical urologists, more studies, with a long follow-up and a large sample size, are needed to clarify the possible additive value of RT-PCR results in staging for localized prostate cancer and predicting disease progression. At present, the decision to perform a radical prostatectomy in a patient with localized prostate cancer should not be influenced by the result of molecular staging.

- Most reports conclude that the RT-PCR assay has a low clinical sensitivity (percentage of patients with metastatic disease who are RT-PCR positive).
- Although PSA and PSM expression is not totally prostate-restricted, in practice, PSA and PSM mRNA detection in the RT-PCR assay seem to be specific for patients with prostate cancer.
- The evaluation of the clinical correlations is difficult due to the lack of standardization and the incompleteness of patient follow-up data in most studies.
- A positive RT-PCR result only indicates the presence of circulating tumor cells in the peripheral blood and does not necessarily imply that metastasis will occur or has occurred.
- Although the biological significance of these prostate cells in the circulation is not known, these patients may represent a subset of patients who will relapse with metastatic disease or suffer tumor recurrence after an initial complete response to therapy.
- PSA and PSM RT-PCR may ultimately be clinically useful; however, as with most medical testing and clinical sensitivity and specificity will not be absolute and will need to be balanced.

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