Circulating Prostate Cancer Cells Detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR): What Do They Mean?

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The clinical application of “molecular staging” of prostate cancer, which uses the reverse transcriptase-polymerase chain reaction to detect cells that contain PSA or PSMA in the bloodstream, is controversial.

**Background:** Molecular techniques have been developed recently to assess for circulating tumor cells. This “molecular staging” of prostate cancer uses the reverse transcription-polymerase chain reaction (RT-PCR) to detect cells that contain PSA or PSMA in the bloodstream. Currently, the clinical application of this concept is controversial.

**Methods:** The authors discuss the current status of molecular biologic methods to detect circulating prostate cancer cells. They report on the limitations of the technology and the advances that will allow the quantification of these circulating cells.

**Results:** Studies generally indicate an increasing level of PSA RT-PCR positivity as disease advances. However, reports have been significantly diverse, and there is no clear explanation for this disparity.

**Conclusions:** The determination of the “circulating prostate cancer cell load” by RT-PCR or other techniques may prove to be useful in the management of patients with prostate cancer, but questions remain to be answered before we can develop and assess new therapeutic strategies that will advance the treatment of prostate cancer before metastasis becomes evident. A better understanding of the biology of tumor cells present in the circulatory system is also needed.

**Introduction**

The clinical significance of circulating prostate cancer cells has long been questioned. In 1961, Jonasson and associates\(^1\) reported transient dissemination of prostate cancer cells into the vena cava as detected by a Papanicolaou stain in patients undergoing a rectal massage or a transurethral resection of the prostate. Interestingly, these cells reached peak detectable levels in the first 10 minutes after the massage and were cleared after 30 minutes. Other circulating cancer cell studies also suggested that these cells perish in the bloodstream. Engell’s colon cancer study\(^2\) in 1955 concluded that approximately 50% of patients surviving five to nine years had venous tumor cells detected at the time of surgery, implicating that most of these cells were destroyed in the circulation.

These early studies were met with skepticism due to a lack of consensus on what constituted a circulating cancer cell. In 1969, the National Cancer Institute reviewed 81 cytologic photographs and concluded that only four fulfilled the criteria of a “suspicious” cell.\(^3\) In 1975, Salsbury\(^4\) conducted a 20-year literature review, and he concluded that it would be doubtful that circulating cancer cells would carry any prognostic value. Hence, this field subsequently remained relatively dormant until the advent of the molecular PCR and RT-PCR technology.

In 1992, our group revisited the concept that circulating prostate cells could be detected using molecular biologic methods. Using RT-PCR targeted at prostate-specific antigen (PSA) mRNA, we collected peripheral blood of men with newly diagnosed metastatic prostate cancer and demonstrated that 38.5% with clinically localized disease were PSA RT-PCR positive with a strong statistical correlation to pathologic stage.\(^5\) Others have not been able to corroborate these staging results.\(^6\)

This review examines controversial issues surrounding the significance of circulating prostate cancer cells and updates investigators on the current biotechnological advances that allow us to detect, quantitate, and isolate circulating prostate cancer cells with unprecedented accuracy. These detection methods may significantly change our understanding and treatment of this disease.

**Epidemiology and Biology of Prostate Cancer Metastasis**

In 1998, approximately 182,000 new prostate cancer cases will be diagnosed, of which approximately 50% will undergo radical prostatectomy based on historic trends.\(^7\) In 22% to 45% of these cases, the operation may fail signaled by a detectable serum PSA, suggesting that up to 41,000 men will harbor persistent cancer.\(^7,8\)

Similarly, men undergoing definitive radiotherapy will likely face similar recurrences. Many patients treated with such curative intent will manifest disease recurrence at distant sites such as bone.

The development of metastasis is a complex process that includes the exodus of tumor cells from the organ, survival of cells in the circulation, and establishment of a metastatic deposit. Bone metastasis is the eventual site of spread in over 85% of cases.\(^9\) Non-osseous cancer can invade the skeleton via three mechanisms: direct extension, retrograde venous flow, and the general arterial circulation.\(^10\) Prostate cancer initially invades the cancellous marrow and, at autopsy, the most common metastatic sites in decreasing order are the spine, femur, pelvis, ribs, sternum, and skull.\(^9,10\) These sites mirror the distribution of active red marrow. Hence, the pattern of prostate cancer bone invasion and distribution is most consistent with dissemination through the systemic arterial system.\(^9,10\) Studies in animals demonstrate that the primary requisite for vertebral metastasis is a hematopoietic marrow with access gained through the arterial system.\(^11,12\)

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**Notes:**

These animal models also require that cells be injected into the left ventricle. This mechanism of tumor dissemination is used due to the fact that 98% of cancer cells injected into the tail vein are cleared in the pulmonary vascular bed in a single pass. Solid tumor animal studies show that at least 10,000 tumor cells may be required for intravenous injection in order to form a single metastatic deposit. Extrapolating this to man, at least 2 cells per mL of blood may be needed in the circulation to form a single metastatic deposit. This is an exquisitely low level of cells to detect by standard pathologic or cytologic means.

These observations led us to develop a method to detect prostate cancer cells in the circulation by using RT-PCR. We demonstrated that prostate cancer cells could be detected in the circulation of patients with metastatic prostate cancer and that women and negative controls failed to detect any prostate cells in the circulation. It should be stressed that these initial observations neither quantitated nor positively characterized the prostate cells as cancerous. However Ts’o and colleagues were recently able to isolate and characterize these as "prostate cancer cells" in the peripheral blood of patients with advanced prostate cancer at a concentration of 1 to 20 cells per 1 mL of whole blood.

The body of literature suggests that when these cells are detected through a properly designed RT-PCR protocol, they are of a malignant phenotype. Since our first 1992 PSA RT-PCR report, there has been a rapid expansion in the literature to use this technique to stage prostate cancer. This attempt to detect cells outside the prostate has been generally referred to as "molecular staging."

### Molecular Staging With RT-PCR

RT-PCR has been used to detect prostate cells in the peripheral blood, lymph nodes, and bone marrow and at the operative field. However, most clinical molecular staging studies have been performed on peripheral blood samples.

The principal steps of molecular staging involve a peripheral phlebotomy, whole blood fractionation, RNA extraction, reverse transcription, PCR amplification, and detection of the PCR products. Approximately 6 to 8 mL of peripheral venous blood is initially subjected to centrifugation on a Ficoll density gradient to isolate mononuclear cells. Our work demonstrated that PSA-expressing cells and mononuclear cells cofractionate in the Ficoll-gradient "buffy coat" in clinical samples from patients. In the latest technique, blood is drawn directly into a CPT Vacutainer tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) that allows fractionation of the blood without a time-consuming Ficoll gradient. Cells of the isolated Buffy coat are lysed and mRNA is isolated by chemical separation using a standard molecular biology technique.

RT-PCR is performed by an automated programmable thermal controller that provides the various temperature conditions necessary for unique primers to select PSA mRNA and generate ampler quantities of cDNA for molecular analysis. Presence of a specific amplified gene product can be examined by agarose gel electrophoresis and can be verified by gene sequencing, Southern blot analysis, or restriction enzyme digest analysis. The amplification of a specific PSA gene product should correlate with the presence of PSA-expressing prostatic cells in the peripheral circulation.

The sensitivity of detection by RT-PCR has been estimated as 1 cell in 10⁷ to 10⁸ mononuclear cells. PSA RT-PCR positivity in metastatic prostate cancer cases ranges between 31% and 88%. In negative normal controls, the false-positive rate has been mostly zero, but recent reports have suggested a range between 10% and 20%, suggesting that these highly sensitive assays may detect a potentially problematic background expression of PSA (M. O’Hara, unpublished data, 1998). It should be noted that primers to prostate-specific membrane antigen (PSMA) have also been used in the RT-PCR assay for prostate cancer, with the results not significantly improved over PSA.

The results of published clinical studies on PSA RT-PCR in the peripheral blood are summarized in the Table. In the first clinical report describing this approach, we examined patients with stage D0-3 (occult, nodal, or bone metastasis) to determine if PSA-expressing cells could be detected in the peripheral venous blood. The RT-PCR assay was positive in one third of the cases (4 of 12 cases), implicating the presence of circulating prostatic cells. In control patients (men with clinically diagnosed benign prostatic hypertrophy or women), the RT-PCR assay was negative (0 of 17 cases).

Using a modified PSA RT-PCR assay, researchers at Columbia University found that all negative controls tested negative, while 14 (77.8%) of the 18 metastatic cases tested positive. More importantly, they found a statistically significant correlation between pathologic stage and PSA RT-PCR that was positive in 25 (38.5%) of the 65 radical prostatectomy cases.

However, other investigators such as Seiden et al and Ghossein et al have failed to correlate PSA RT-PCR with pathologic stage with metastatic cases testing...
positive in 31% to 35% of cases. On the other end of the spectrum, Sokoloff et al. found 88% PSA RT-PCR positivity in metastatic cases, but the 59% positive rate in organ-confined patients was excessively high. Based on 82 consecutive radical prostatectomies, Ignatoff et al. concluded that PSA RT-PCR correlated weakly with serum PSA, Gleason score, and pathologic stage. However, they found a strong positive rate in metastatic cases and a nearly perfect negative correlation in normal controls.

Overall, most studies report a 0% PSA RT-PCR rate in negative control cases, while the positive rate in the metastatic group is much higher -- between 31% and 88%. Indeed, there is an increase PSA RT-PCR positivity rate with advancing stage from T1-2 to T3-4 and on up to metastatic disease. However, most reports do not yet recommend PSA RT-PCR as a practical staging tool. Olsson et al. claim that a positive PSA RT-PCR is a significant predictor of recurrence-free survival time. In their series from Columbia University, 14 (7%) of 190 PSA RT-PCR-negative patients had a serum PSA greater than 0.2 ng/mL, within five years after radical prostatectomy compared with 36 (28%) of 127 PSA RT-PCR-positive patients. Last, it is possible that a combination of primaries may improve the overall staging accuracy of RT-PCR. Preliminary work from the Cleveland Clinic suggests that combining RT-PCR for PSA and PSA-MMA may improve the staging accuracy.

Despite some of these promising reports, PSA RT-PCR has fallen short of being predictive enough to be used at the bedside. Several factors have contributed to the inconsistent PSA RT-PCR reports in peripheral blood. Sample handling and timing of the blood draw is critical. Ubiquitous nucleases in our environment make mRNA exquisitely susceptible to degradation such that the starting sample could already be compromised. There have been suggestions in the literature that a variety of surgical procedures ranging from biopsies to radical prostatectomies may lead to hematogenous dissemination of prostatic cells. In an analysis of the effect of transrectal ultrasound prostate biopsy, we found a 9% seroconversion rate, ie, 9% of patients who had no detectable PSA-expressing cells in their peripheral circulation prior to surgery tested PSA RT-PCR postoperatively. In a separate study, a significant portion of patients (25%) seroconverted to a positive RT-PCR at three months after surgery.

Another paramount factor is assay technical variability, eg, primer selection, PCR product detection methods, and cross contamination, all of which affect sensitivity and specificity. Even in ideal conditions, if 10 identical PCR reactions are performed in parallel and simultaneously, there can be a threefold difference in the quantity of PCR end products. In a comparison of three independent RT-PCR protocols, Slawin and associates found a large degree of variation in the detectability of PSA in peripheral blood of patients with clinically localized disease. Most PCR experts agree that in a strict sense, PCR reactions cannot be precisely quantitated, which may be the Achilles' heel of this assay.

Technical issues outlined here indicate that an accurate assessment of the clinical utility of the RT-PCR assay requires strict quality control standards and specifications at multiple levels ranging from selection criteria of patients, sample acquisition, storage and processing, and a detailed analysis of RT-PCR assay conditions. Optimization of all parameters from specimen collection and handling through the RT-PCR protocol is currently the focus of several studies. For example, investigators at Baylor College reported that PSA RT-PCR predicated pathologic stage when done postoperatively compared with preoperatively. Ylikoski and colleagues have recently presented a quantitative PSA RT-PCR assay that ambitiously aims to target one of the significant weaknesses of this system. Our group has participated in a multi-institutional consortium that aims to address many of these difficult technical issues; slow but steady progress is being made.

**Isolation, Quantitation, and Characterization of Circulating Prostate Cancer Cells**

Both flow cytometry and now RT-PCR have allowed these cells to be discovered in the peripheral circulation. Further quantification and characterization of these cells represent the next challenge in determining the significance of these circulating cells. Ts'o et al at Johns Hopkins University recently isolated and characterized prostate cancer cells from the peripheral blood of eight men with hormone-refractory metastatic prostate cancer. Cells were isolated by fractionating the whole blood over a Percoll gradient, and the isolated cells were identified with PSA immunofluorescent staining and fluorescent in situ hybridization. Approximately 1 to 20 prostate cancer cells per 1 mL of blood were identified with positive PSA cytologic staining and chromosome 7 and 8 aneuploidy, which is common for prostate cancer cells but rare for lymphocytes. Similarly, Moss et al. reported positive PSA staining cells in fractionated whole blood from prostate cancer patients, which correlated with positive PSA RT-PCR results but not with clinical stage.

Using a combination of density gradient and magnetic cell sorting, Brandt et al. identified 10 to 100 prostate cancer cells per 40 mL (both single cells and in clusters) in 10 prostate cancer patients. Positive isolation and identification were performed with cytokeratin and PSA antibodies.

Racila et al. reported on a new immunomagnetic enrichment system by which epithelial cancer cells can be isolated, identified, and quantitated in breast cancer and in a limited number of prostate cancer patients. Unlike magnetic bead technology, these workers used a magnetic ferrofluid that may be more effective at capturing cells present at low concentrations. Immunomagnetic separation of epithelial cells from peripheral blood is remarkably fast and simple to perform. Target epithelial cells are captured by nanometer-size iron particles (ferrofluid) linked to an epithelial cell-specific antibody when whole blood is incubated with this ferrofluid. This mixture is subjected to magnetic separation at room temperature. The whole epithelial enrichment process takes one hour and requires no centrifuges or special equipment outside of the proprietary magnet (Immuneon Corp, Huntington Valley, Pa). This assay can detect and enumerate 1 epithelial cell in 1 mL of whole human blood with flow cytometry performed on the ferrofluid enriched specimen. Peripheral blood from 30 breast and 3 prostate cancer patients contained a substantially greater number of circulating epithelial cells compared with 13 normal controls. These epithelial cells stained positive with cytokeratin and maein-1, and their morphology was consistent with malignancy. Preliminary work by these investigators has shown a correlation between quantity of circulating prostate cancer cells and disease progression (L. Terstappen, unpublished data, 1998). A newer system under development will allow these cells to be captured with the ferrofluid and directly observed under a fluorescent microscope, thus eliminating the need for a costly flow cytometer.

**Conclusions**

A growing body of evidence is now validating the existence and importance of circulating prostate cancer cells. These cells were initially detected grossly by flow cytometry and now by RT-PCR. Further enhancements in cell capture may allow quantification and further characterization of these cells that are attempting to establish a metastatic lesion. It is clear that even with PSA, Gleason grade, and sophisticated imaging tools, we are unable to clinically distinguish curable organ-confined prostate cancer from non-organ-confined incurable disease. Since our initial report on the use of PSA RT-PCR for detecting circulating prostate cancer cells, several studies have attempting to use this assay to define pathologic organ confined from non-organ confined disease. Most studies have indicated an increasing level of PSA RT-PCR positivity as one advances in stage. However, reports have been widely disparate, and there is no one clear explanation for this disparity. Many other solid tumors (breast, melanoma, colon cancer, and others) are also being studied using RT-PCR technology. Workers in a number of disciplines studying these different solid tumors may unravel the intricacies of these important new technologies.

Important questions regarding the biology of cells in the circulatory system will need to be answered. In addition, a quantitative reproducible assay needs to be developed that can specifically detect those prostate cancer cells harboring the machinery that will enable them to survive the circulation and grow at a distant site. Once these two important issues are resolved, we may then have a powerful tool to develop and assess new therapeutic strategies and to enable us to rapidly
advance the treatment of prostate cancer in much the same way human immunodeficiency virus treatment has been advanced with sophisticated blood tests that measure the viral load. One day it may be possible to quantify a circulating prostate cancer cell load. This determination will allow therapeutic decisions long before the PSA shows its characteristic and ominous elevation in patients long before metastasis become evident.

Dr Gomella and Dr Moreno have joint patent rights with Thomas Jefferson University to some RT-PCR technology.

References


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