

Review

Molecular Detection of Micrometastases and Circulating Tumor Cells in Solid Tumors

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Abstract

The detection of circulating tumor cells and micrometastases may have important prognostic and therapeutic implications. Because their numbers can be very small, these tumor cells are not easily detected using conventional methods. In the last decade, molecular techniques have been widely used for the detection of occult tumor cells. The objective of this report is the application of these molecular tools to solid tumors. A systematic review of all related English-language articles published in the last 32 years was performed. The molecular detection of occult tumor cells can be accomplished by PCR amplification of tumor-specific abnormalities present in the DNA or mRNA of malignant cells. The other main PCR strategy for the detection of CTC and micrometastases involves amplification of tissue-specific mRNA. This latter method was often applied to solid tumors, whereas the former was occasionally used. PCR was shown to be superior to conventional techniques in detecting occult tumor cells, allowing the identification of 1 malignant cell mixed with 1 to 10 million normal cells. In some reports, PCR is shown to be a strong predictor of outcome. The molecular detection of circulating tumor cells and micrometastases in solid tumors can be accomplished using highly sensitive PCR assays. The central question of whether PCR reliably predicts relapse and survival remains unanswered for many types of solid tumor. If PCR-based assays are found to be a reliable tool, they will likely have a major impact on the management of these malignancies.

BACKGROUND

The first publication on CTCs² is attributed to Ashworth (1), who in 1869 described a case of cancer in which cells

similar to those in the tumor were found in the blood after death. Occasional reports on the subject were published until 1955 when Engell (2) captured the imagination of many researchers by reporting the detection of CTCs in advanced cancer patients using a cell block technique. This article generated great enthusiasm in the research community (3). Between 1955 and 1965, 5000 cancer patients were tested for circulating tumor cells by 40 investigative teams using 20 different cytological methods (3). The initial studies reported a very high positivity rate among cancer patients (up to 100%; Ref. 3). However, these results were soon shown to be invalidated by the fact that circulating hematopoietic elements, especially megakaryocytes, were often confused with tumor cells. When cell preservation techniques were improved allowing a better morphological analysis, the detection of true CTCs by light microscopy was shown to have a very low sensitivity (~1%) in cancer patients (3). Consequently, routine cytological examination of blood specimens for CTCs was abandoned. The issue of CTCs and MRD resurfaced 20 years later with the advent of immunohistochemistry. Sensitive immunocytological tests were developed to detect tumor cells in the BM and PB of patients with neuroblastoma, breast carcinoma, and lung carcinoma (4–6). These tests were shown to identify BM disease with much greater sensitivity than conventional techniques (5–6). Indeed, these immunocytological tests were said to detect a single tumor cell seeded among 10,000–100,000 mononuclear cells. Despite evidence of the prognostic value of this determination in some studies (6–9), the detection of micrometastases by immunocytochemistry was not routinely used in cancer staging protocols (10). This was due to a combination of factors, such as the absence of clinical significance in some studies (11–14), loss of antigen expression in poorly differentiated tumors, reports of cytokeratin and epithelial membrane antigen positivity in nonepithelial cells (15, 16), and the hope for the development of an ever better method for the detection of occult tumor cells using nucleic acid analysis. This hope has been materialized by the advent of the highly sensitive PCR technique in the late 1980s that has greatly facilitated the detection of occult tumor cells. Since 1987, a variety of PCR-based techniques have been devised for the identification of CTCs and micrometastases in leukemias, lymphomas, melanoma, neuroblastoma, and various types of carcinomas (17–22).

PCR TECHNOLOGY

PCR is an *in vitro* method that enzymatically amplifies specific DNA sequences using oligonucleotide primers (short DNA sequences composed of 18–25 nucleotides in length) that flank and therefore define the region of interest in the target DNA (23). The procedure consists of a repetitive series of cycles, each of which consists of template denaturation, primer annealing, and extension of the annealed primers by a thermostable DNA polymerase to create the exponential accumulation of a specific DNA fragment, the ends of which are determined

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² The abbreviations used are: CTC, circulating tumor cell; MRD, minimal residual disease; BM, bone marrow; PB, peripheral blood; RT-PCR, reverse transcription-PCR; PSA, prostate-specific antigen; PC, prostatic carcinoma; PSMA, prostate-specific membrane antigen; CEA, carcinoembryonic antigen; AJCC, American Joint Committee on Cancer; EWS, Ewing's sarcoma; AFP, α -fetoprotein; TGB, thyroglobulin; TPO, thyroid peroxidase; *PTI*, prostate tumor inducing gene.

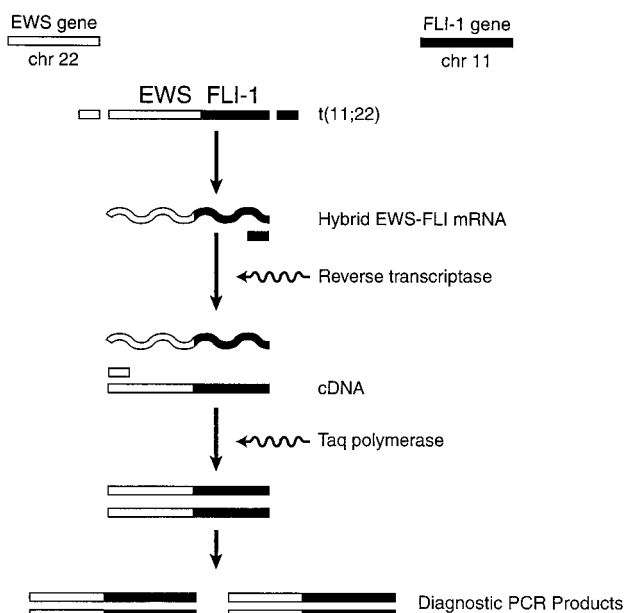


Fig. 1 Detection of occult tumor cells by RT-PCR amplification of tumor-specific abnormalities in the mRNA. In this example, the primers are chosen to flank the t(11;22) translocation present in EWS. This translocation juxtaposes the *FLI-1* gene on chromosome 11 to the *EWS* gene on chromosome 22. The primers will therefore anneal to and amplify the hybrid EWS/*FLI-1* transcript when the translocation is present.

by the 5' ends of the primers (23). After 20 cycles, the amplification is about 10^6 – 10^8 -fold (23). PCR amplification can be accomplished using RNA as starting material. This procedure, known as RT-PCR is similar to DNA PCR with the modification that PCR amplification is preceded by reverse transcription of RNA into cDNA.

One major strategy for the detection of occult tumor cells is the PCR amplification of tumor-specific abnormalities present in the DNA or mRNA of these cells. This approach is mostly used for the detection of MRD in hematological malignancies. It was first applied to the detection of the t(14;18) translocation associated with follicular lymphomas (21). The primers used hybridize to the region flanking the translocation and will therefore only amplify the DNA when the translocation is present. If the translocation is not present, the primers anneal to different chromosomes, and PCR is impossible. RT-PCR amplification of tumor-specific mRNA was first used for the detection of the t(9;22) translocation in chronic myelogenous leukemia (24). The detection of occult tumor cells by RT-PCR of chimeric tumor-specific mRNA has been performed in a few solid tumors such as EWS (Ref. 25; Fig. 1).

The other main PCR strategy for the detection of occult tumor cells involves amplification of tissue-specific mRNA by RT-PCR. This has been mainly used for the detection of CTCs and micrometastases in solid tumors (Table 1). This approach is based on the fact that malignant cells often continue to express markers that are characteristic or specific of their tissue of origin. PSA, for example, has become a useful marker for the detection and monitoring of prostatic carcinoma. In principle,

Table 1 RT-PCR and DNA PCR methods for the detection of occult tumor cells in solid tumors

Tumor type	Molecular target
Melanoma	Tyrosinase mRNA MART 1 mRNA
Prostate	PSA mRNA PSMA mRNA <i>PTI-1</i> mRNA ^a
Breast carcinoma	Muc 1 mRNA CEA mRNA Cytokeratin 19 mRNA
Hepatocellular carcinoma	AFP mRNA Albumin mRNA
Gastrointestinal carcinomas	K-ras mutations ^a CEA mRNA
Neuroblastoma	Tyrosine hydroxylase mRNA PGP 9.5 mRNA GAGE mRNA ^a
EWS	EWS/ <i>FLI1</i> fusion transcript ^a EWS-ERG fusion transcript ^a
Uterine cervix carcinoma	SCC antigen mRNA HPV E6 mRNA ^a
Thyroid carcinomas of follicular origin	TGB mRNA TPO mRNA

^a Except for these molecules, all other markers are tissue specific. PGP 9.5, neuroendocrine protein gene product; SCC, squamous cell carcinoma; HPV, human papilloma virus.

PCR amplification of tissue-specific mRNA offers several advantages over the protein-based assay: (a) RNA is very unstable in the extracellular environment; therefore, its detection should indicate the presence of tumor cells in the examined tissue or body fluid; (b) although monoclonal antibody tests are becoming increasingly sensitive, they are not expected to approach the single-molecule detection capability of PCR tests; (c) tissue-specific mRNA can indicate the presence of tumor cells despite a negative protein-based assay. For example, PSA mRNA transcripts have been detected in poorly differentiated prostatic carcinoma cells that do not express the PSA protein (26). From a technical standpoint, RT-PCR detection of any tissue-specific marker requires knowledge of its gene sequence and specifically of intron-exon junctions, which facilitates the selection of oligonucleotide primers for RT-PCR (Fig. 2).

LIMITATIONS OF PCR TECHNOLOGY

False-Positive PCR Results. The power of PCR resides in the extreme sensitivity of the technique; current publications report the detection of 1 tumor cell diluted with 1–10 million normal cells (Fig. 3; Refs. 20 and 27). It is this extreme sensitivity that confers an inherent tendency to produce false-positive results if sufficient precautions are not taken to prevent contamination of samples. One study reported a wide variability of results from one laboratory to the next using coded samples (28). Meticulous laboratory techniques have been developed to prevent contamination of samples (23). False-positives could be due to the general process of illegitimate transcription (*i.e.*, transcription of any gene in any cell type). Although the number of these transcripts in inappropriate cells is very low (estimated at one mRNA molecule per 100–1000 cells; Ref. 29), it can result in the occurrence of false-positives because of the high

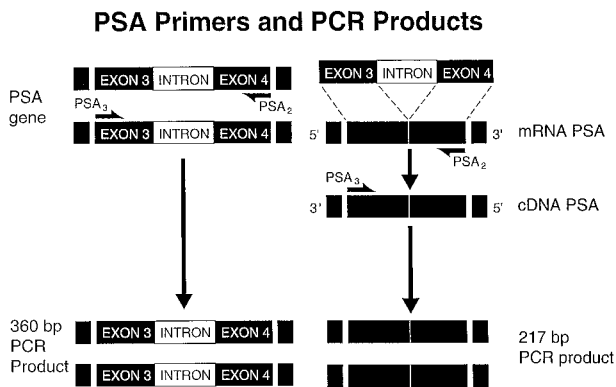


Fig. 2 Detection of occult tumor cells by RT-PCR of tissue-specific mRNA. In this example, primer sets for PSA mRNA were selected to span the intronic sequence. This allowed discrimination, based on size, between RT-PCR products from mRNA targets (*right*) and PCR products from contaminating genomic DNA (*left*). [From Ghossein, R., and Rosai, J. PCR in the detection of micrometastases and circulating tumor cells. *Cancer* (Phila.), 78: 10–16, 1996. Copyright 211 (1996) American Cancer Society. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.]

sensitivity of RT-PCR. For example, a neuron-specific marker, neuroendocrine protein gene product 9.5, was shown to be present in scant amounts in normal bone marrow cells (30). However, even in the presence of such a phenomenon, it is possible to design specific RT-PCR assays by optimizing the PCR thermocycling conditions, as has been shown for tyrosinase mRNA, a marker of melanocytic lineage (31). Pseudogenes can also give rise to false-positive results. Because they lack an intronic sequence, RT-PCR amplification of pseudogenes will lead to PCR products indistinguishable from those generated from the mRNA. Current RT-PCR tests for the detection of CTCs and micrometastases are limited by lack of tissue-specific markers in many solid tumors. Consequently, false-positive results will necessarily occur if nonspecific markers such as albumin, epithelial membrane antigen, or estrogen receptors are tested in control samples. This is especially the case when these poorly specific markers are tested in complex tissues containing a variety of cell types, such as BM and lymph nodes (32). Because most markers of CTCs and micrometastases in solid tumors are tissue specific (*i.e.*, expressed in tumor and their normal tissue of origin), the mechanical introduction of normal or benign cells in the circulation after invasive procedures may lead to false-positive PCR results. For example, many studies showed that a significant number of patients converted from RT-PCR negative to RT-PCR positive after radical prostatectomy (33). These studies were performed on peripheral and operative field blood samples. However, the percentage of RT-PCR-negative patients hemoconverting after less invasive procedures (*e.g.*, transrectal ultrasound, prostatic core biopsy) was much lower. These false-positive PCR results can be averted by scheduling the RT-PCR tests several weeks after any invasive procedure. We did not encounter false-positive results while PCR testing PB and BM for melanocytic tissue-specific markers in our control population (34). Our control group included dark-skinned individuals, making it unlikely that venipuncture

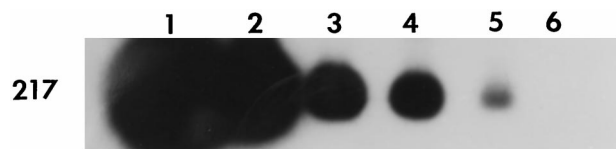


Fig. 3 RT-PCR for PSA mRNA followed by Southern blot hybridization of the RT-PCR products. Results of dilutions of prostatic carcinoma cells (LNCaP) with peripheral blood mononuclear cells: *Lane 1*, 1:100; *Lane 2*, 1:1,000; *Lane 3*, 1:10,000; *Lane 4*, 1:100,000; *Lane 5*, 1:1,000,000; *Lane 6*, negative control. *Left*, diagnostic fragment (bp). RT-PCR can detect one LNCaP tumor cell diluted with 1 million peripheral blood mononuclear cells. (From Ghossein, R., *et al.* Detection of circulating tumor cells in patients with localized and metastatic prostatic carcinoma: clinical implications. *J. Clin. Oncol.*, 13: 1195–1200, 1995. Reprinted by permission of W. B. Saunders Company.)

by itself is a cause of false-positives by RT-PCR. In our studies, the lack of false-positives with tyrosinase RT-PCR is probably due to the fact that PCR sensitivity *in vivo* is not as high as the one reported *in vitro* (see next paragraph). PCR is therefore not able to detect the rare normal melanocytes that are introduced in the sample after blood drawing or BM aspiration.

False-Negative PCR Results. The sensitivity of PCR is variable, and this can lead to false-negative results, especially in the detection of occult tumor cells, where low level signals are expected. Inhibitors present in some tissues and fluids can diminish PCR sensitivity. Therefore, careful controls are necessary to ensure that there is amplifiable RNA or DNA in the sample. This is accomplished by demonstrating amplification of a constitutively present transcript such as actin. The reader should therefore be aware that the *in vitro* sensitivity reported in all articles on CTCs and micrometastases (often expressed in number of cell line-derived tumor cells detected per million of white cells) does not reflect the *in vivo* sensitivity of PCR. The latter is most probably lower than the *in vitro* sensitivity because of inhibitors of the PCR reaction present in tissues and body fluids and because the tumor cell line chosen for these sensitivity experiments strongly express the marker of interest. In contrast, the tumor cells *in vivo* do not necessarily express the marker of interest because of tumor cell heterogeneity. False-negatives could also be due to technical errors (*e.g.*, omission of one of the reagents) or to a sampling problem because only a few milliliters of peripheral blood are analyzed at a certain time. The latter could be due to intermittent shedding of tumor cells in the circulation. This problem should be overcome by sequential sampling, defined as the analysis of multiple blood samples at different time points. False-negative results could also be due to down-regulation of the target gene by therapy (*e.g.*, hormonal treatment) or to the presence of poorly differentiated subclones that do not express the tissue-specific marker being tested. For example, PSA mRNA expression was shown to be decreased by antiandrogen therapy (35) and in poorly differentiated prostatic carcinoma (26). The use of multiple markers will help diminish these types of false-negatives.

Quantitative PCR. It is possible to quantify the amount of target nucleic acids present in a given sample using RT-PCR. This can be done by the addition of differently sized competitor target molecules (36–38) or the use of serial dilutions (39).

Table 2 RT-PCR detection of CTC and BM micrometastases in PC using PSA and PSMA mRNA

Author (Ref.)	Marker	Sample	RT-PCR positive/Total (%)	
			Localized PC ^a	Metastatic PC ^b
Katz <i>et al.</i> (42)	PSA mRNA	Blood	25/65 (38%)	14/18 (78%)
Israeli <i>et al.</i> (41)	PSA mRNA	Blood	0/18 (0%)	6/24 (25%)
	PSMA mRNA	Blood	13/18 (72%)	16/24 (67%)
Seiden <i>et al.</i> (43)	PSA mRNA	Blood	3/41 (7%)	11/35 (31%)
Ghossein <i>et al.</i> (27)	PSA mRNA	Blood	4/25 (16%)	26/76 (34%)
Sokoloff <i>et al.</i> (44)	PSA mRNA	Blood	43/69 (62%)	29/33 (88%)
	PSMA mRNA	Blood	12/69 (17%)	13/33 (39%)
Corey <i>et al.</i> (45)	PSA mRNA	Blood	12/63 (19%)	6/13 (46%)
		BM	45/63 (71%)	10/13 (77%)
Wood and Banerjee (46)	PSA mRNA	BM	39/86 (45%)	

^a Localized PC includes stage A, B (clinically organ-confined disease only).

^b Metastatic PC includes stage D1–D3 patients (D1, pelvic lymph node metastases; D2, distant metastases without prior hormonal therapy; D3, D2 disease refractory to hormonal therapy) in all the listed studies except in that of Israeli *et al.* (41). In that article (41), three patients with D0 disease (elevated serum tumor markers only) were also included as metastatic PC.

These quantitative PCR methods are, however, unable to estimate the number of tumor cells present in a sample, because the transcription rate (*i.e.*, the amount of target mRNA) varies between individual tumor cells (40). This fact significantly limits the value of quantitative PCR in detecting occult tumor cells.

APPLICATIONS TO SPECIFIC TUMOR TYPES

Prostatic Carcinoma. PCR detection of CTCs has the potential to improve case selection in patients with localized PC and to monitor disease activity more accurately in patients with metastatic disease. We and others have detected circulating tumor cells in the peripheral blood of patients with localized and metastatic PC using RT-PCR for PSA mRNA (Refs. 27 and 41–46; Table 2). We detected circulating tumor cells in 16% of patients with clinically organ confined (T₁₋₂) disease and in 35% of patients with distant metastases (27). In accordance with most other reports on the subject (33), none of our controls were positive, indicating the specificity of the technique when applied to peripheral blood. The frequency of positivity increases with tumor stage and high serum PSA levels. A disappointment was that a significant proportion of patients with metastatic disease was negative. Prostatic cells may be shed intermittently in the circulation; this phenomenon leads to sampling errors. Another possibility is the presence in the circulation of tumor cells that express very low levels of PSA mRNA because they are poorly differentiated or down-regulated by hormonal therapy.

Katz *et al.* (42) showed that the presence of circulating tumor cells correlated with both capsular penetration and positive surgical margins (42). Their RT-PCR test was found to be superior to other staging modalities in predicting pathological stage. Therefore, these authors propose the use of this test as a staging modality for radical prostatectomy candidates (“molecular staging”). Other authors have not found a statistically significant correlation between RT-PCR positivity and capsular penetration (44). Clearly, additional studies are needed to assess the staging capability of this test in localized PC. With regard to molecular prognosis, some groups have found a statistically significant correlation between preoperative RT-PCR positivity for PSA mRNA in peripheral blood and postoperative biochem-

ical failure (Ref. 47; Table 3). In a recent study, we analyzed the PB of 122 men with metastatic, androgen-independent PC for the presence of PSA mRNA. Sixty-four of these patients were tested in our institution, whereas the others were assayed at the Dana-Farber Cancer Institute. We found that RT-PCR positivity correlates with decreased overall survival in both institutions. We also showed that RT-PCR is superior to a single serum PSA measurement in predicting survival in both groups of patients (48).

RT-PCR for PSA mRNA has also been used to detect occult tumor cells in lymph nodes and BM of patients with PC (45, 46, 49–51). This technique was shown to be more sensitive than immunohistochemistry and standard histopathology in detecting lymph node micrometastases in localized disease (49). Wood and Banerjee (46) followed 86 patients with clinically localized disease in whom preoperative bone marrow PSA RT-PCR was performed. These authors defined recurrence as a postoperative serum PSA > 0.4 ng/ml or clinical evidence of locally recurrent disease by digital rectal examination. Four % of the RT-PCR-negative patients recurred after prostatectomy, whereas 26% of the RT-PCR-positive patients failed postoperatively (46). Edelstein *et al.* (51) found a similar correlation when they studied pelvic lymph nodes using RT-PCR for PSA mRNA (51). Thirty % of the PCR-negative patients failed, compared with 87.5% of the PCR-positive patients within a 5-year follow-up period (51).

Recently, RT-PCR assays for two additional prostatic markers, PSMA and prostate tumor inducing gene (*PTI-1*), have been reported (41, 52, 53). PSMA is a cell surface protein with sequence homology to transferrin, found to be expressed in poorly differentiated PC cells (54), hormone refractory cells (55), and bone metastases (56). PSMA transcripts were detected in the peripheral blood of patients with localized and metastatic PC using RT-PCR (41, 43, 52). *PTI-1* is a novel oncogene that was cloned from a LNCaP cDNA library (57). Using RT PCR, Sun *et al.* (53) were able to detect *PTI-1* mRNA in the PB of patients with metastatic PC.

Breast Carcinoma. The majority of mammary carcinoma patients (~90%) present with tumors that are clinically confined to the breast and neighboring axillary lymph nodes.

Table 3 Molecular prognosis in PC using RT-PCR for PSA

Failure was defined as serum PSA ≥ 0.2 ng/ml after radical prostatectomy (RP) in the article by Olsson *et al.* (47) and serum PSA >0.4 ng/ml or local recurrence on digital rectal exam after RP in the article by Wood and Banerjee (46). In both of these articles, patients were sampled for RT-PCR before RP. Although no RR was given in the article by Wood and Banerjee (46), RT-PCR positivity did correlate with poorer failure-free survival. Only those articles using Kaplan-Meier survival analysis are included in this table.

Author (Ref.)	Patient population	Sample	End point	Univariate RR ^a	P
Olsson <i>et al.</i> (47)	Localized PC	Blood	Failure-free survival	3.6	0.0286
Wood and Banerjee (46)	Localized PC	BM	Failure-free survival	NA	0.004
Ghossein <i>et al.</i> (48)	Metastatic AI PC	Blood	Overall survival	2.25	0.028

^a RR, relative risk; NA, not available; AI, androgen independent.

Essentially, all of these patients are rendered free of measurable disease after primary surgery (58). Despite this highly efficient locoregional therapy, 30–40% of these patients will develop metastatic disease within 10 years if no further treatment is instituted (58). The chief reason for this therapy failure is that breast carcinoma cells disseminate throughout the body early in tumor development (59). To prevent the clinical progression of these micrometastases, about two-thirds of the patients diagnosed with stage I to III breast cancer are candidates for adjuvant or neoadjuvant chemotherapy (60). To better identify those patients who will benefit from adjuvant chemotherapy, several groups have attempted the detection of BM micrometastases by immunohistochemistry (7, 61, 62). Some authors have indicated the prognostic significance of these sensitive immunocytochemical assays (7, 62), but others failed to demonstrate such relevance (11–14). Indeed, a significant minority of patients whose BM was positive by immunohistochemistry have remained free of clinically evident metastatic disease after a long follow-up period. These findings could be due to several factors. Some micrometastases may be incapable of developing into clinically significant lesions (58). Alternatively, the antibodies may have cross-reacted with normal marrow elements, leading to false-positive results.

Several authors were able to detect tissue-specific transcripts in the PB, BM, and lymph nodes of patients with breast carcinomas using highly sensitive RT-PCR assays (20, 63–69). Noguchi *et al.* (68) reported the detection of muc1 mRNA (a core protein of polymorphic epithelial mucin) in all lymph nodes histologically positive for metastatic mammary carcinoma and in none of the control lymph nodes. Furthermore, muc1 mRNA was detected in 6 of 41 lymph nodes immunohistochemically negative for polymorphic epithelial mucin (an epithelial marker; Ref. 68). Unfortunately, a recent article revealed the presence of Muc1 mRNA in the blood of healthy donors and in lymph nodes of patients without cancer (70). This finding limits the value of Muc1 as a marker of occult tumor cells (70). CEA mRNA was detected by RT-PCR in the PB, BM, and lymph nodes of breast cancer patients (20, 69). According to these authors, their benign controls were all negative. Other groups, however, found CEA RT-PCR to be nonspecific (see “Gastrointestinal Carcinoma”). Using RT-PCR, Fields *et al.* (63) were able to identify cytokeratin 19 mRNA in the BM of stage II–IV breast carcinoma patients (63). According to their study, the presence of these transcripts by RT-PCR correlates with poor prognosis in patients with metastatic breast cancer undergoing high-dose chemotherapy and autologous BM trans-

plantation (63). The value of these findings is, however, limited by the low specificity of this marker. Indeed, RT-PCR for cytokeratin 19 mRNA was positive in 20% of healthy volunteers in one study (71). Clearly, there is a need for more specific markers of occult disease in breast carcinoma.

Malignant Melanoma. The main current criteria to assess prognosis in malignant melanoma are the histopathological features of the primary tumor and the clinical presentation. However, these factors are of limited value, especially in the advanced stages of the disease (72). There is, therefore, a need for better prognostic markers in those patients. The molecular detection of CTCs and BM micrometastases has the potential of predicting outcome in patients with malignant melanoma. Smith *et al.* (31) were the first to propose that melanoma cells could be detected in the PB using RT-PCR for tyrosinase mRNA (31). Tyrosinase is a key enzyme in melanin biosynthesis that catalyzes the conversion of tyrosine to dopa and of dopa to dopaquinone. This test is presumed to detect circulating melanoma cells because tyrosinase is one of the most specific markers of melanocytic differentiation (73), and melanocytes are not known to circulate. Furthermore, most studies show that tyrosinase mRNA is not present in the PB of healthy individuals (34, 72, 74–76). Since the original study of Smith *et al.* (31), several groups have attempted the detection of CTCs in malignant melanoma using tyrosinase mRNA (34, 72, 74–80). As shown in Table 4, the PCR positivity rates are extremely variable, ranging from 0 to 100%. These discrepant results could be in part explained by differences in RNA extraction and PCR methodology (73). These disparate findings could also be due to unrecognized contamination, leading to false-positive results (73). Indeed, Foss *et al.* (77) acknowledged the presence of significant technical problems due to carry over contamination that took 1 year to overcome. Despite these discrepancies, we and others have shown that RT-PCR for tyrosinase mRNA in PB is able to predict overall survival and disease-free survival in a statistically significant manner (34, 72, 75, 79, 80; Table 5). We were also able to specifically detect tyrosinase transcripts in the BM of patients with AJCC stages II–IV melanoma (stage II, primary tumor >1.5 mm in thickness with no metastases; stage III, regional lymph node metastases; stage IV, distant metastases; Ref. 34). In patients with thick and advanced melanoma who were rendered surgically free of disease (AJCC stages II–IV), we found that BM positivity for tyrosinase mRNA is an independent predictor of poorer disease-free survival (81). In this same patient population (median follow-up of 18.3 months), blood RT-PCR positivity for tyrosinase was shown to be an

Table 4 Detection of CTCs in the peripheral blood of patients with cutaneous malignant melanoma using RT-PCR

Author (Ref.)	No. of RT-PCR-positive patients/Total no. of patients tested (%) according to AJCC stage ^a		
	I-II	III	IV
Brossart <i>et al.</i> (76)	1/10 (10%)	6/17 (35%)	29/29 (100%)
Hoon <i>et al.</i> (74) ^b	13/17 (76%)	31/36 (86%)	63/66 (95%)
Battayani <i>et al.</i> (72)	2/10 (20%)	22/51 (43%)	16/32 (50%)
Foss <i>et al.</i> (77)			0/6 (0%)
Pittman <i>et al.</i> (78)			3/24 (12.5%)
Kunter <i>et al.</i> (75)	0/16 (0%)	0/16 (0%)	9/34 (26%)
Mellado <i>et al.</i> (79)	14/39 (36%)	7/17 (41%)	33/35 (94%)
Curry <i>et al.</i> (80) ^c	48/160 (30%)	60/116 (52%)	

^a Stage I, primary tumor <1.5 mm in thickness with no metastases; stage II, primary tumor >1.5 mm in thickness with no metastases; stage III, regional lymph node metastases; stage IV, distant metastases.

^b In this study, the peripheral blood was analyzed for four markers (tyrosinase, p97, Muc 18, and MAGE-3).

^c In this report, the samples were tested for tyrosinase and MART-1. In both articles (74, 80), RT-PCR positivity was defined as positivity for any of the markers. In all the other studies listed, tyrosinase alone was used as a marker for melanoma cells.

independent predictor of poorer overall and disease-free survival (81). These observations may have important clinical implications. RT-PCR may help define subsets of patients with poor prognosis for whom toxic forms of adjuvant therapies are justified. This test may help improve the stratification of patients for clinical trials into more homogeneous groups. This assay could also be used to measure treatment response in patients on current or novel therapeutic regimens like vaccine therapy.

The presence or absence of lymph node metastases is a powerful predictor of survival in patients with malignant melanoma. Standard histopathological interpretation routinely underestimates the number of patients with lymph node metastases (82). Indeed, routine histological examination samples at most 1% of the submitted tissue. Immunohistochemical staining with antibodies against S-100 protein or HMB-45 melanoma antigen increases the yield of occult lymph node metastases (82). However, because of the rate-limiting step related to the number of sections that need to be examined, these techniques have not been used widely. To circumvent these problems, Wang *et al.* (82) attempted the detection of lymph node micrometastases using RT-PCR for tyrosinase mRNA and showed this technique to be superior to immunohistochemistry or morphology. Sentinel lymph node biopsy is an alternative to elective dissection or observation for managing lymph node basins in patients with cutaneous melanomas. Several groups, including ours, are currently testing sentinel lymph nodes for the presence of tyrosinase by RT-PCR with the hope that this technique will help better stratify patients for elective lymphadenectomy. In our laboratory, we were able to detect tyrosinase mRNA by RT-PCR in 73% of sentinel lymph nodes from patients at risk for regional nodal metastases, including all of those with histologically positive sentinel lymph nodes and 65% of the histologically negative specimens (83). Two of 18 control nodes without melanoma were tyrosinase PCR positive (83). We are presently following up with these patients to assess the prognostic value of this assay.

Gastrointestinal Carcinoma. As with other solid tumors, the detection of early metastatic spread in gastrointestinal malignancies may help stratify patients for radical surgery and guide adjuvant therapies. Several authors reported the detection

of CEA mRNA in the PB, BM, and lymph nodes of patients with gastric, colorectal, and pancreatic carcinomas but in none of the control subjects (20, 69, 84–86). CEA mRNA was detected by RT-PCR in lymph nodes and BM specimens that were negative by immunohistochemistry for CEA and cytokeratin (20, 86). In patients with Tumor-Node-Metastasis stage II colorectal carcinomas (who have no lymph node metastases), the detection of CEA mRNA in regional lymph nodes was shown to correlate with a poorer 5-year survival rate (86). However, in some studies, CEA mRNA was detected by RT-PCR in lymph nodes and BM samples from individuals without epithelial malignancies (87, 88). A different approach was used by Hardingham *et al.* (89) to detect CTCs in colorectal cancer patients. In this assay, tumor cells are captured using immunomagnetic beads and then subjected to a PCR-restriction enzyme digestion procedure that identifies *K-ras* gene mutations (89). The authors found *K-ras* mutations in the PB of 9 of 27 colorectal carcinoma patients but in none of the control samples. PCR was predictive of relapse in their study (89).

Small Round Cell Tumors of Infancy and Childhood (Neuroblastoma and EWS/Peripheral Neuroectodermal Tumor). Evaluation of BM metastases is a routine and important component of clinical staging in patients with neuroblastoma (19). Therefore, the availability of a sensitive assay to detect occult neuroblastoma cells in BM specimen would be highly desirable. Using RT-PCR for tyrosine hydroxylase mRNA (an enzyme involved in catecholamine biosynthesis), Miyajama *et al.* (19) were able to detect neuroblastoma cells in the PB and BM of patients with organ-confined disease and lymph node metastases. Another interesting application of RT-PCR for occult neuroblastoma cells resides in the detection of residual tumor in stem cell grafts. Lode *et al.* (90) were able to detect neuroblastoma cells in stem cell grafts using a combination of tyrosine hydroxylase RT-PCR and antiganglioside immunocytochemistry (90). The probability of event-free survival was lower in the group with contaminated stem cell grafts compared with the group with no detectable contamination (90). However, this difference was not statistically significant. Recently, GAGE mRNA was detected in the PB of 50% of patients with widespread neuroblastoma (91). GAGE belongs to a family of tumor-

Table 5 Molecular prognosis in melanoma using RT-PCR

The relative risk was not available in all the above references. However, in each article RT-PCR positivity correlated with poorer survival time. Only those articles using Kaplan-Meier survival analysis are included in this table. In the article by Curry *et al.* (80), the samples were tested for tyrosinase and Mart 1. RT-PCR positivity was defined as positivity for any of the markers.

Author (Ref.)	AJCC stage ^a	Marker	Sample	End point	P
Mellado <i>et al.</i> (79)	II–III	Tyrosinase	Blood	DFS	<0.05
Kunter <i>et al.</i> (75)	IV	Tyrosinase	Blood	OS	≤0.0006
Ghossein <i>et al.</i> (34)	II	Tyrosinase	Blood	OS	0.01
	II	Tyrosinase	BM	OS	0.06
	III	Tyrosinase	Blood	OS	0.02
Curry <i>et al.</i> (80)	I–III	Tyrosinase/Mart 1	Blood	DFS	0.0022

^a Stage I, primary tumor <1.5 mm in thickness with no metastases; stage II, primary tumor >1.5 mm in thickness with no metastases; stage III, regional lymph node metastases; stage IV, distant metastases.

^b DFS, disease-free survival; OS, overall survival.

associated antigens that is expressed in various types of human tumors, including neuroblastoma, but is highly restricted among normal tissues except for the adult testis (92). The clinical significance of these findings is, however, unknown.

As mentioned previously in this section, residual occult tumor cells present in autologous marrow grafts were thought (for many years) to be the cause of relapse after autologous BM transplantation in many tumor types including neuroblastoma (93, 94). Gene marking provided evidence in support of this theory. Bone marrow grafts of neuroblastoma patients were retrovirally transduced at the time of harvest with the neomycin-resistance gene. In that study, gene-marked neuroblastoma cells were found in all patients who relapsed (94). Analysis of neuroblastoma DNA suggested that at least 200 tumor cells were introduced with the autologous marrow graft and contributed to the relapse (94).

Despite its usual presentation as a localized solid tumor, ES of bone or soft tissue is believed to be a systemic disease at the time of diagnosis (25). EWS and the closely related neoplasm peripheral neuroectodermal tumor contain the t(11;22)(q24;q12) translocation in 90% of the cases (25), which is thought to be specific for this family of tumors. This rearrangement juxtaposes the *EWS* gene on chromosome 22 to the *FLI-1* gene on chromosome 11 (25). Several authors were able to detect the fusion mRNA (*EWS/FLI-1*) by RT-PCR in the PB and BM of patients with ES and peripheral neuroectodermal tumor (25, 95, 96). Fagnou *et al.* (97) found that the detection of the *EWS/FLI-1* or its variant translocation *EWS/ERG* mRNA in BM is a predictor of overall survival in univariate analysis. In another study, the detection of *EWS*-related transcript was not predictive of early relapse (98). The true biological and clinical significance of these results requires large-scale prospective studies.

Other Solid Tumors. Because hepatocytes are known to produce albumin and AFP, several groups have attempted the detection of these transcripts in the PB of hepatocellular carcinoma patients (22, 99, 100). AFP mRNA was detected by RT-PCR in 36% of hepatocellular carcinoma patients, including individuals with no clinical evidence of metastatic tumor (22). The value of this test is, however, limited by the presence of AFP mRNA in the PB of patients with nonneoplastic liver disease (cirrhosis and chronic hepatitis; Refs. 99 and 101). RT-PCR for albumin mRNA was shown to be even less specific (102). In one study, all normal individuals had albumin mRNA in their blood (99).

The transcript of the squamous cell carcinoma antigen, a serine protease inhibitor released from malignant and nonma-

lignant squamous cells, was detected by RT-PCR in the PB of patients with squamous cell carcinomas of the uterine cervix (103). Another group attempted the detection of CTC in squamous cell carcinoma of the uterine cervix using RT-PCR for the human papilloma virus type 16 *E6*-transforming gene mRNA (104). In this study, RT-PCR for human papillomavirus *E6* mRNA was present in the PB of 12 of 13 patients with distant metastases (104).

On the basis of tissue specificity, TGB and TPO mRNA were used as markers for circulating thyroid tumor cells (105–107). In one study, both TGB and TPO transcripts were found in 13 of 24 (54%) patients with thyroid carcinoma (106). TGB mRNA was detected in 4 of 20 (20%) patients with benign thyroid nodules, whereas TPO mRNA was found in 3 (15%) of these cases (106). All normal individuals were negative for TPO and TGB mRNAs in their PB. In this study, there was a statistically significant correlation between the presence of TGB and TPO transcripts in the PB and a diagnosis of thyroid carcinoma (106). The prognostic significance of these findings is unknown.

Accurate staging of head and neck squamous cell carcinoma is critical because it will determine treatment modalities. Current standard histopathological techniques may not detect small numbers of tumor cells at the resection margins or in cervical lymph nodes. In an attempt to better stage patients with head and neck carcinoma, Brennan *et al.* (108) used sequencing and molecular probing to detect *p53* gene mutations in histologically negative surgical margins and cervical lymph nodes. These authors found *p53* mutations in 6 of 28 histologically negative lymph nodes (21%) and in a significant number of margins that were negative for carcinoma by light microscopy (108). Patients with positive margins by molecular assays appear to have a substantially increased risk of recurrence. This approach is limited, however, by the fact that only 50% of head and neck carcinoma patients harbor *p53* mutations, and sequencing is technically cumbersome (109). Recent technological advances in automated sequencing and probing and the use of multiple genetic markers will help overcome these limitations.

CONCLUSION AND FUTURE TRENDS

Over the last few years, the advent of the highly sensitive PCR technique has greatly facilitated the detection of occult tumor cells in many forms of cancer. PCR has been shown to be superior to conventional techniques in detecting CTCs and micro-

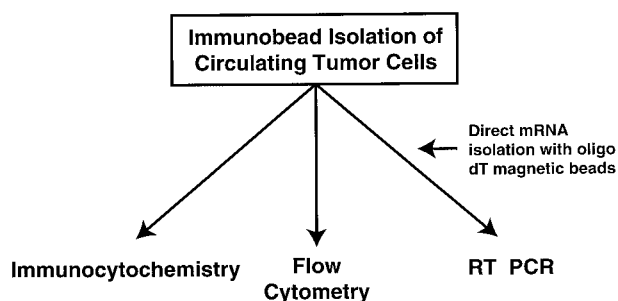


Fig. 4 Immunobead-based assays for the detection of CTCs. The tumor cells are first captured using magnetic beads coated with an antibody directed against a specific cell type (e.g., Ber-EP4 anti-epithelial cell antibody against carcinomas). The CTCs are then lysed, and their mRNA is isolated using oligo(dT) magnetic beads. This preparation is then ready for RT-PCR. The isolated magnetic cell fraction can also be subjected to flow cytometry and immunocytochemistry.

metastases in both hemato-lymphoid and epithelial malignancies. The presence of CTCs or micrometastases does not necessarily lead to the development of clinically significant metastatic disease. Several authors have shown that the metastatic process in humans and animals is inefficient, both on a temporal and a numerical basis (110, 111). The vast majority of tumor cells that enter the bloodstream are killed by mechanical, immunological, and unknown mechanisms (110, 112). However, as stated by Weiss (110), total metastatic inefficiency is seldom (if ever) complete, and metastases are the major cause of death in patients with cancer. In the past decade, several studies have shown that PCR detection of occult disease has prognostic significance in some solid tumors (see Tables 3 and 5). However, the central question of whether PCR positivity reliably predicts relapse in an independent manner remains unanswered in many types of solid tumors. Analysis of a large number of patients by well-qualified laboratories is needed and is currently undertaken in many institutions. In this regard, the sensitivity and predictive value of PCR-based assays for the detection of occult tumor cells could be improved by serial sampling and the use of multiple markers. Sampling at different time points was shown to increase the prognostic value of PCR-detected MRD in hematological malignancies (17). The development of a specific multiple marker PCR assay increases the sensitivity of this technique by overcoming the problem of tumor cell heterogeneity.

Because of the limitations of PCR (e.g., contamination of samples, inability to quantify tumor cells or perform functional assays), it is now clear that other techniques should be used as an adjunct for the detection of occult tumor cells. In the past year and a half, we and others have used immunomagnetic separation technology as a means to improve the detection of CTCs (113–115). In this technique, the specimen is incubated with magnetic beads coated with antibodies directed against epithelial cells. The epithelial cells are then isolated using a powerful magnet. The magnetic fraction can be used for downstream RT-PCR, flow cytometry, or immunocytochemical analysis (Fig. 4). The sample used for RT-PCR will, therefore, be considerably enriched in tumor cells with a minimal background of nonneoplastic cells. In addition, oligo (dT)-coated magnetic beads can be used to isolate mRNA. The use of mRNA instead of total RNA as starting material decreases background, result-

ing from unspecific priming, and helps avoid the inhibitory effects due to high concentrations of total RNA. Epithelial cell enrichment and mRNA isolation using magnetic beads will render RT-PCR much more sensitive and specific. Multicolor flow cytometry and immunocytochemical analysis of the specimen will allow quantification of the tumor cells (114) and their assessment for various markers of disease progression. The clinical value of occult tumor cell detection will be greatly enhanced by the combined use of RT-PCR, flow cytometry, and immunocytochemistry.

If these assays for CTCs and micrometastases are found to reliably predict relapse and death, this will likely have a major impact on the treatment of many solid tumors. Patients could be selected for systemic therapy at an earlier stage when the metastatic tumor burden is low (116). These techniques may improve the preoperative staging of patients with epithelial malignancies and therefore help avoid unnecessary radical procedures. Furthermore, these tests may be useful in monitoring the effectiveness of novel or current adjuvant therapy, the intensity and duration of which is tailored to the individual patient. The molecular detection of circulating tumor cells and micrometastases is likely to have a profound impact in the management of patients with solid tumors.

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