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Molecular detection and characterisation of circulating tumour cells and micrometastases in solid tumours

R.A. Ghossein *, S. Bhattacharya

Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY, 10021, USA

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Abstract

The detection and molecular characterisation of circulating tumour cells (CTC) and micrometastases may have important prognostic and therapeutic implications. Because their numbers are very small, these tumour cells are not easily detected using conventional methods. In the last decade, numerous groups have attempted to detect occult tumour cells in solid malignancies using the highly sensitive reverse transcriptase polymerase chain reaction (RT–PCR). These assays were in the vast majority directed against tissue-specific markers. PCR was shown to be superior to conventional techniques in detecting occult tumour cells allowing the identification of one malignant cell mixed with 1–10 million normal cells. In some tumours like melanoma and prostatic carcinoma, tissue-specific transcripts were detected with high specificity in the blood of patients with localised and advanced disease. In some reports, PCR was shown to be a strong predictor of poorer outcome. However, due to the many limitations of PCR (e.g false-positives), many groups are developing new approaches for the detection of occult tumour cells. The most attractive technique involves immunomagnetic isolation of CTC and micrometastases prior to downstream analysis. The tumour-rich magnetic fraction can be subjected to RT–PCR, immunocytochemistry and *in situ* hybridisation. This will lead to better quantification and molecular characterisation of these tumour cells. In conclusion, the molecular detection and characterisation of occult tumour cells offer a great opportunity for better stratifying patients with solid tumours and for developing new prognostic markers and targeted therapies.

Keywords: Minimal residual disease; Reverse transcriptase; Polymerase chain reaction; Molecular diagnostics

1. Introduction

The detection of circulating tumour cells (CTC) has interested researchers and physicians since 1869 when Ashworth described a case of cancer in which cells similar to those in the tumour were found in the blood after death [1]. The detection of CTC gained great attention in 1955 when Engell reported the detection of CTC in patients with various types of carcinoma using a cell block technique [2]. Indeed, between 1955 and 1965, several thousand cancer patients (most with solid malignancies) were tested for CTC 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%) [3]. However, these results were soon shown to be due to false-positives since circulating haematopoietic elements, especially megakaryocytes, were often confused with tumour cells. When cell preservation techniques were improved allowing a better morphological analysis, the detection of true CTC by light microscopy was shown to have a very low sensitivity (approximately 1%) in cancer patients [3]. Routine cytological examination of blood specimens for CTC was therefore abandoned in 1965. The issue of CTC and micrometastases resurfaced 20 years later with the advent of immunocytochemistry. Sensitive immunocytological tests were developed to detect tumour cells in the bone marrow (BM) and peripheral blood (PB) of patients with neuroblastoma, breast and lung carcinomas [4–6]. These tests were shown to identify BM micrometastases with much greater sensitivity than conventional techniques [5,6]. Indeed, these immunocytological assays were said to detect a single tumour cell seeded amongst 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 immunocyto-

* Corresponding author. Tel.: +1-212-639-2701; fax: +1-212-717-3203.

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chemistry 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 tumours and reports of cytokeratin and epithelial membrane antigen positivity in non-epithelial cells [15,16]. Meanwhile, there was the prospect of developing an even better method for the detection of occult tumour cells using nucleic acid analysis. This hope materialised with the advent of the highly sensitive polymerase chain reaction (PCR) technique in the late 1980s that has greatly facilitated the detection of occult tumour cells. Since 1987, a variety of PCR-based techniques have been devised for the identification of CTC and micrometastases in leukaemias, lymphomas and various types of solid malignancies [17–22]. In this article, we will focus on the detection and characterisation of CTC in five major types of solid tumours, namely melanoma and carcinomas of the prostate, breast, lung and gastrointestinal tract.

2. 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 whose ends are determined by the 5′ ends of the primers [23]. After 20 cycles , the amplification is approximately 10^6–10^8-fold [23]). PCR amplification can be accomplished using RNA as starting material. This procedure is known as reverse transcriptase PCR (RT–PCR). It 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 tumour cells is the PCR amplification of tumour-specific abnormalities present in the DNA or mRNA of these cells. This approach has mostly been used in haematological malignancies. It was first applied to the detection of the t(14;18) translocation associated with follicular lymphomas [21]. The primers used hybridise 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 no PCR product is obtained. The detection of occult tumour cells by RT–PCR of chimeric tumour-specific mRNA has been performed in leukaemias [24] and in a few solid tumours such as Ewing's sarcoma [25] (Fig. 1).

The other main PCR strategy for the detection of occult tumour cells involves amplification of tissue-specific mRNA by RT–PCR. This has been mainly used for the detection of CTC and micrometastases in solid tumours since tumour-specific abnormalities are rare in non-haematopoietic malignancies (Table 1). This approach is based on the fact that malignant cells often continue to express markers that are characteristic of or specific to the normal tissue from which the tumour originates or with which the tumour shares histotype. It is the appearance of these tissue-specific mRNAs at a body site where these transcripts are not normally present that implies tumour spread (e.g. *prostate-specific antigen* (*PSA*) mRNA in BM). It is important to mention that in principle PCR amplification of tissue-specific mRNA offers several advantages over the protein-based assays. Firstly, RNA is very unstable in the extracellular environment; therefore its detection should indicate the presence of tumour cells in the examined body fluid or tissue. Although monoclonal antibodies are becoming increasingly sensitive, they are not expected to approach the single-molecule detection capability of PCR tests. Thirdly, tissue-specific mRNA can be present despite a negative protein-based assay. For example, PSA mRNA transcripts have been detected in poorly differentiated prostate carcinoma cells that do not express the PSA protein [26]. From a technical standpoint, RT–PCR detection of any tissue-specific

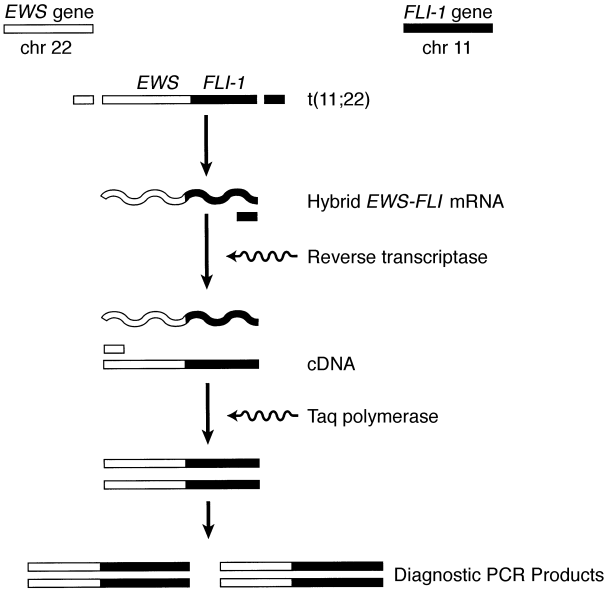

Fig. 1. Detection of occult tumour cells by reverse transcriptase–polymerase chain reaction (RT–PCR) amplification of tumour specific abnormalities in the mRNA. In this example, the primers are chosen to flank the t(11;22) translocation present in Ewing's sarcoma (EWS). This translocation juxtaposes the *FLI-1* (Friend leukaemia virus integration site 1) gene on chromosome 11 to the Ewing's sarcoma (*EWS*) gene on chromosome 22. The primers will therefore anneal to and amplify the hybrid *EWS*/*FLI*-1 transcript when the translocation is present. (Reproduced with permission from Ghossein and colleagues [25a].

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).

2.1. Limitations of PCR technology

2.1.1. False-positive PCR results

The power of PCR resides in the extreme sensitivity of the technique, current publications have reported the detection of 1 tumour cell per 1 ml of whole blood (Fig. 3) [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 [29]) it can result in the occurrence of false-positives because of the high sensitivity of RT–PCR. For example, a neuronal specific marker, neuroendocrine protein gene product (PGP 9.5) was shown to be present in scant amount in normal BM cells [30]. In view of this problem, there has been great effort to find genes that display the least amount of illegitimate transcription in blood, BM and lymph nodes [31]. Some authors have attempted to solve this issue by optimising the PCR thermocycling conditions, as has been shown for *tyrosinase* mRNA, a marker of melanocytic lineage [32]. For example, the number of PCR cycles should be carefully selected to be high enough to detect occult tumour cells but low enough to avoid amplification of illegitimate transcripts [31]. Processed pseudogenes can also give rise to false-positive results. Since they lack an intronic sequence, RT–PCR amplification of processed pseudogenes will lead to PCR products indistinguishable from those generated from the mRNA. Current RT–PCR tests for the detection of CTC and micrometastases are limited by lack of tissue-specific markers in many solid tumours. Consequently, false-positive results will necessary occur if non-specific markers such as albumin, epithelial membrane antigen or oestrogen receptors are tested in control samples. Because most markers of CTC and micrometastases in solid tumours are tissue-specific (i.e. expressed in the tumour 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 haemoconverted from RT–PCR-negative to RT–PCR-positive after radical prostatectomy [33]. However, the percentage of RT–PCR-negative patients haemoconverting after less invasive procedures (e.g. transrectal ultrasound, prostatic core biopsy) was much lower. These false-positive PCR results can be averted by timing the RT–PCR tests so they are carried out weeks after any invasive procedure. In principle, venipuncture by itself may generate false-positives because of the introduction of normal keratinocytes or melanocytes in the circulation. 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 is a cause of false-positives by RT–PCR. This 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. In order to avoid this problem, some authors recommend discarding the first few millilitres of

Table 1
RT–PCR and DNA PCR methods for the detection of occult tumour cells in solid tumours

Tumour type	Molecular target
Melanoma	*Tyrosinase* mRNA
	MART 1 mRNA
	GAGE mRNA*
Prostate	*PSA* mRNA
	PSMA mRNA
	PTI-1 mRNA*
Breast carcinoma	*Muc* 1 mRNA
	CEA mRNA
	Cytokeratin-19 mRNA
Hepatocellular carcinoma	*AFP* mRNA
	Albumin mRNA
Gastrointestinal carcinomas	*CEA* mRNA
	Cytokeratin 20 mRNA
Lung carcinoma	*CEA* mRNA
	Muc 1 mRNA
	Cytokeratin-19 mRNA
	Surfactant protein mRNA
Neuroblastoma	*Tyrosine hydroxylase* mRNA
	PGP 9.5 mRNA
	GAGE mRNA*
Ewing's sarcoma	*EWS/FLI1* fusion transcript*
	EWS-ERG fusion transcript*
Uterine cervical carcinoma	*SCC antigen* mRNA
	Human papilloma virus (*HPV*) *E6* mRNA*
Thyroid carcinomas of follicular origin	*TGB* mRNA
	TPO mRNA

RT–PCR, reverse transcriptase–polymerase chain reaction; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; PTI-1, prostate tumour-inducing gene; CEA, carcinoembryonic antigen; Muc 1, mucin 1; AFP: alpha feto protein; PGP 9.5, neuroendocrine protein gene product; EWS, Ewing's sarcoma; FLI-1, Friend leukaemia virus integration site 1; ERG, erythroblastosis virus-transforming sequence related gene; SCC, squamous cell carcinoma; HPV, human papilloma virus; TGB, thyroglobulin; TPO, thyroid peroxidase. Except for those molecules labelled with *, all other markers are tissue-specific.

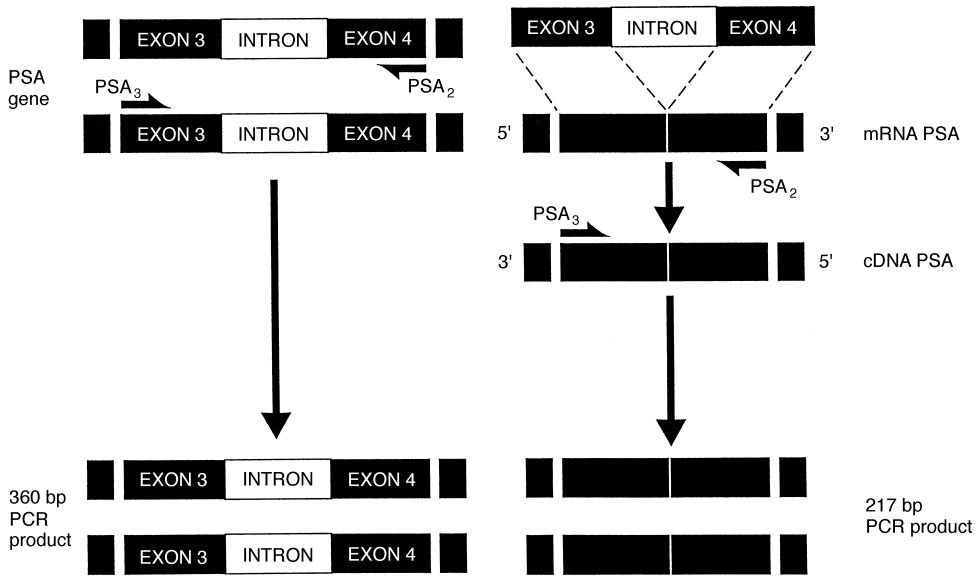

Fig. 2. Detection of occult tumour cells by reverse transcriptase–polymerase chain reaction (RT–PCR) of tissue-specific mRNA. In this example, primer sets for prostate specific antigen (PSA) mRNA were selected to span the intronic sequence. This will allow discrimination, based on size, between RT–PCR products from mRNA targets (right) and PCR products from contaminating genomic DNA (left). Taken from [26a]. Copyright © (1996) American Cancer Society. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

blood that are collected and may be contaminated with normal cells from the epidermis.

2.1.2. False-negative PCR results

The sensitivity of PCR is variable, and this can lead to false-negative results, especially in the detection of occult tumour 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 CTC and micrometastases (often expressed as the number of cell line-derived tumour cells detected per million 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 tumour cell line chosen for these sensitivity experiments strongly expresses the marker of interest. In contrast, tumour cells *in vivo* do not necessarily express the marker of interest because of tumour cell heterogeneity. False-negatives could also be due to technical errors (e.g. omission of one of the reagents), to a sampling problem or to intermittent shedding of tumour cells into the circulation since only a few millilitres of PB are analysed at a certain time. The latter two problems could be minimised by sequential sampling, defined as the analysis of multiple blood samples at different time-points. False-negative results could also be due to downregulation 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 anti-androgen therapy [35] and in poorly differentiated prostatic carcinoma [26]. In this setting, a multiple marker PCR assay may help increase PCR positivity by overcoming the problem of tumour cell heterogeneity.

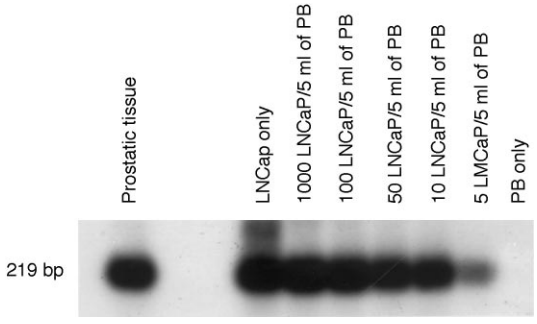

Fig. 3. Immunobead nested reverse transcriptase polymerase chain reaction (RT–PCR) for prostate-specific membrane antigen (PSMA) mRNA after Southern blot hybridisation of the nested RT–PCR products. Results of sensitivity experiment. Samples containing only prostatic tissue and LNCap prostatic carcinoma cells are used as positive controls. A sample containing only peripheral blood (PB) from a healthy subject is used as negative control. The remaining samples are serial dilutions of LNCaP cells with whole blood from healthy volunteers. After nested immunobead RT–PCR (two rounds of amplification), PCR can produce a band corresponding to 5 LNCaP cells diluted in 5 ml of PB. The diagnostic fragment is indicated on the left in base-pairs. (Reproduced by permission of Lippincott Williams and Wilkins [27].)

2.1.3. Quantitative PCR

It is now possible to quantify the amount of target nucleic acids present in a given sample with a user-friendly automated real-time quantitative RT–PCR assay [36]. These quantitative PCR methods are, however, unable to estimate the number of tumour cells present in a sample, since the transcription rate (i.e. the amount of target mRNA) varies between individual tumour cells [37]. This fact limits the value of quantitative PCR in detecting occult tumour cells.

3. Applications to specific tumour types

3.1. Prostatic carcinoma

RT–PCR detection of CTC and micrometastases has the potential to improve case selection in patients with localised prostatic carcinoma (PC) and to monitor disease activity more accurately in patients with metastatic disease. We and others have detected occult tumour cells in the PB and BM of patients with localised and metastatic PC using RT–PCR for PSA and (PSMA) mRNA [38–47] (Table 2). We detected CTC in 16% of patients with clinically organ-confined (T1–2) disease and in 34% of patients with distant metastases [38]. 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 PB. The frequency of RT–PCR positivity increases with tumour stage and high serum PSA levels [38]. Unfortunately, a significant proportion of patients with metastatic disease were negative. Prostatic cells may be shed intermittently in the circulation and this phenomenon leads to sampling errors. Other possibilities include: (1) the presence in the circulation of tumour cells that express very low levels of *PSA* mRNA because of tumour cell heterogeneity; and (2) a difference in sensitivity between different sets of PCR primers for a given marker [50,51]. In order to avoid false-positives due to the mechanical introduction of benign prostatic epithelial cells into the circulation, our patient population was tested eight weeks after any prostatic invasive procedure. One article reported the detection of CTC in 20% of previously RT–PCR-negative patients after needle biopsy [52]. The conversion rates were similar in patients regardless of biopsy results. Testing of serial postbiopsy samples revealed that most patients haemoconverting after biopsy reverted to a RT–PCR-negative PCR assay within 4 weeks [52].

Two groups of researchers showed that the presence of CTC by RT–PCR correlated with both capsular penetration and positive surgical margins [40,53]. They found RT–PCR to be superior to other staging modalities in predicting pathological stage and proposed the use of this test as a staging modality for radical prostatectomy candidates. Other well-conducted studies have not found a statistically significant correlation between blood RT–PCR positivity and pathological stage [42,45]. Clearly, additional studies are needed to assess

Table 2
RT–PCR detection of circulating tumour cells and bone marrow micrometastases in prostate carcinoma (PC) using *PSA* and *PSMA* mRNA

Author [Ref.]	Marker	Sample	RT–PCR positive/total (%)	
			Localised PC[a] *n* (%)	Metastatic PC[b]
Katz [40]	*PSA* mRNA	Blood	25/65 (38)	14/18 (78)
Israeli [39]	*PSA* mRNA	Blood	0/18	6/24 (25)
	PSMA mRNA	Blood	13/18 (72)	16/24 (67)
Seiden [41]	*PSA* mRNA	Blood	3/41 (7)	11/35 (31)
Ghossein [38]	*PSA* mRNA	Blood	4/25 (16)	26/76 (34)
Sokoloff [42]	*PSA* mRNA	Blood	43/69 (62)	29/33 (88)
	PSMA mRNA	Blood	12/69 (17)	13/33 (39)
Corey [43]	*PSA* mRNA	Blood	12/63 (19)	6/13 (46)
	PSA mRNA	BM	45/63 (71)	10/13 (77)
Wood [44]	*PSA* mRNA	BM	39/86 (45)	–
Gao [45]	*PSA* mRNA	Blood	25/84 (30)	3/8 (38)
Ennis [46]	*PSA* mRNA	Blood	55/201 (27)	–
Loric [48]	*PSMA* mRNA	Blood	6/17 (35)	28/33 (85)
Zhang [49]	*PSMA* mRNA	Blood	11/48 (23)	10/11 (91)
	PSA mRNA	Blood	6/48 (13)	7/11 (64)
	PSA/PSMA	Blood	14/48 (29)	11/11 (100)

RT–PCR, reverse transcriptase-polymerase chain reaction; CTC, circulating tumour cells; BM, bone marrow; PC, prostate carcinoma; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen.

[a] Localised PC includes TNM stages I and II (clinically organ-confined disease only).

[b] Metastatic PC includes TNM stages IV patients (N1, pelvic lymph node metastases; M1, distant metastases) in all the listed studies except in that of Israeli and colleagues [39]. In that article [39], 3 patients with so-called D0 disease (elevated serum tumour markers only) were also included as metastatic PC.

the staging capability of this test in localised PC. With regard to molecular prognosis, some groups have found a statistically significant correlation between preoperative RT–PCR positivity for *PSA* mRNA in PB and postoperative biochemical failure [54,55] while other authors did not [45] (Table 3). We analysed the PB of 122 men with metastatic androgen-independent PC for the presence of *PSA* mRNA. 64 of these patients were tested in our institution while the remainder 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 [57].

RT–PCR for *PSA* mRNA has also been used to detect occult tumour cells in lymph nodes and as stated earlier in BM of patients with PC [43,44,58–60]. This technique was shown to be more sensitive than immunohistochemistry and standard histopathology in detecting lymph node micrometastases in localised disease [58]. All control lymph nodes and BM tested negative for *PSA* RT–PCR [33,43,60]. Wood and Banerjee followed 86 patients with clinically localised disease in whom preoperative bone marrow *PSA* RT–PCR was performed [44]. 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 per cent of the RT–PCR-negative patients recurred after prostatectomy, while 26% of the RT–PCR-positive patients failed postoperatively [44]. Edelstein and colleagues found a similar correlation when they studied pelvic lymph nodes using RT–PCR for *PSA* mRNA [60]. Thirty per cent of the PCR-negative patients failed compared with 87.5% of the PCR-positive patients within a 5-year follow-up period [60].

In the last few years, RT–PCR assays for two additional prostatic markers, PSMA and prostate tumour-inducing gene (*PTI-1*), have been reported [39,45,61,62]. PSMA is a cell-surface protein with sequence homology to transferrin. PSMA is expressed in benign and malignant prostatic epithelium [63]. It is upregulated in hormone-refractory states, in metastatic situations or other situations where there is tumour recurrence or extension [63]. *PSMA* transcripts were detected in the peripheral blood of patients with localised and metastatic PC using RT–PCR [39,42,48,56,61]. Some investigators reported a high PCR positivity rate for PSMA mRNA in the blood of healthy individuals [64]. We and others did not encounter any false-positives with *PSMA* RT–PCR [27,49]. When a combined *PSA* and *PSMA* RT–PCR test was used for CTC, this resulted in an increase in sensitivity and prognostic significance compared with a one-marker assay in metastatic androgen-independent PC (R. Ghossein, Memorial Sloan-Kettering Cancer Center, NY, USA). Okegawa and associates showed that a preoperative *PSMA* RT–PCR in blood is capable of predicting biochemical recurrence after radical prostatectomy [56]. *PTI-1* is a novel oncogene that was cloned from a LNCaP cDNA library [65]. Using RT–PCR, Sun and colleagues were able to detect *PTI-1* mRNA in the PB of patients with metastatic PC [62]. Since this original article, no additional studies on PB RT–PCR for *PTI-1* have been reported.

At the present time, RT–PCR assays for CTC and micrometastases in PC are not yet ready for use in the clinical setting. However, these assays are still promising and well worth pursuing.

3.2. Breast carcinoma

The majority of mammary carcinoma patients (approximately 90%) present with tumours that are clinically confined to the breast and neighbouring axillary lymph nodes. Essentially, all these patients are rendered free of measurable disease after primary surgery [66]. Despite this highly efficient loco-regional therapy, 30–40% of these patients will develop clinically detectable metastases within 10 years if no further treatment is instituted [66]. The chief reason for these relapses is that breast carcinoma cells disseminate throughout the body early in tumour development [67]. In order to prevent

Table 3
Molecular prognosis in prostate carcinoma (PC) using RT–PCR for PSA and PSMA[a]

Author [Ref.]	Patient population	Sample	Marker	Endpoint	*P* value
de la Taille [55]	Localised PC	Blood	*PSA*	Failure-free survival	0.0002
Wood [44]	Localised PC	Bone marrow	*PSA*	Failure-free survival	0.004
Gao [45]	Localised PC	Blood	*PSA*	Failure-free survival	0.598
Okegawa [56]	Localised PC	Blood	*PSMA*	Failure-free survival	< 0.01
Ghossein [57]	Metastatic AIPC	Blood	*PSA*	Overall survival	0.028

AI, androgen-independent; RT–PCR, reverse transcriptase polymerase chain reaction; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen.

[a] Failure was defined as serum PSA ⩾ 0.2 ng/ml on one occasion after radical prostatectomy (RP) in de La Taille's [55] article and on two occasions in Gao's article [45]. Recurrence was defined as serum PSA ⩾ 0.4 ng/ml in Okegawa's article [56] and serum PSA > 0.4 ng/ml or local recurrence on digital rectal exam. after RP in Wood's article [44]. In these articles [44,45,55,56] patients were sampled for RT–PCR before RP. Except for Gao's article [45], RT–PCR positivity did correlate with poorer survival. Only those articles using Kaplan–Meier survival analysis are included in this table.

the clinical progression of these micrometastases, approximately two-thirds of the patients diagnosed with stages I to III breast cancer are candidates for adjuvant or neoadjuvant chemotherapy [68]. It has been reported that approximately 36% of these women would remain free of disease using loco-regional therapy alone [68]. Routine adjuvant chemotherapy would subject these patients to unnecessary and toxic treatment. In order to better identify those patients who will benefit from adjuvant chemotherapy, several groups have attempted the detection of BM micrometastases by immunohistochemistry [7,69,70]. Some authors have indicated the prognostic significance of these sensitive immunocytochemical assays [7,70], 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 relatively long intervals [66]. These findings could be due to several factors. Some micrometastases may be incapable of developing into clinically significant lesions [66]. Alternatively, the antibodies may have cross-reacted with normal marrow cells, 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 [20,71–80]. Unfortunately almost all of the markers used were shown to have false-positives (Table 4) [79–85]. These false-positives could be due to illegitimate transcription, the presence of pseudogene or sample contamination. In the hope of improving RT–PCR specificity, several authors have attempted variations of the already published RT–PCR protocols. Slade and colleagues designed a quantitative RT–PCR assay test that defined an upper limit for the background *CK 19* transcripts [88]. These cut-off points were based on the analysis of samples from control individuals. These authors found abnormally high levels of *CK 19* mRNA in the PB of 55% of metastatic breast cancer patients and in 13% of patients with primary tumour (i.e. no metastatic disease on routine bone and liver scans). High *CK 19* transcripts were found in 61% of BM samples from primary breast carcinomas as defined in their study [88]. The clinical impact of their findings is unknown because of a lack of follow-up. Clearly, there is a need for more specific PCR markers in breast carcinoma.

Table 4
RT–PCR and PCR positivity rate in control subjects using putative markers for breast carcinoma[a]

Author [Ref.]	Marker	Sample	Positive *n* (%)
Eltahir [81]	*Muc* 1 mRNA	Bl	21/23 (91)
	CD44 variant	Bl	4/10 (40)
Krismann [83]	*CK 19*	Bl	13/65 (20)
Mori [77]	CEA	Bl	0/22
Lopez-Guerrero [84]	*CK 19*	Bl	0/10
	CEA	Bl	0/4
	Maspin	Bl	1/5 (20)
De Graaf [85]	*EGP-2*	Bl	10/10 (100)
Ko [82]	*CEA*	Bl	8/24 (33)
Zach [86]	*Mammaglobin*	Bl	0/27
Soria [87]	*Telomerase*	Bl	0/9
Bostick [80]	*Beta 1 — 4GalNAc-T*	LN	0/10
	C-Met	LN	1/10 (10)
	P97	LN	1/10 (10)

RT–PCR, reverse transcriptase–polymerase chain reaction; CK, cytokeratin; CEA, carcinoembryonic antigen. Bl, blood; LN, lymph nodes; Beta 1 — 4GalNAc-T, beta 1 — 4-*N*-acetylgalactosaminyltransferase; Muc 1, mucin 1; CTC, circulating tumour cells.

[a] In this table, control subjects were defined as healthy volunteers only in all studies except Lopez-Guerrero's article. In this article [88], control subjects were defined as "healthy volunteers and patients without any type of solid tumours". In Soria's article, telomerase activity was recorded by PCR after immunomagnetic isolation of the CTC. In all other articles, non-immunobead RT–PCR techniques were used.

Mammaglobin is a recently described tissue-specific marker that has homology with a family of secreted proteins that includes rabbit uteroglobin. This marker was found to be present only in adult mammary tissue and in 80–95% of primary breast carcinomas where it is frequently overexpressed [89]. According to one study, this marker is present by RT–PCR in breast carcinoma cell lines and absent in 20 normal lymph nodes [78]. In a small group of breast carcinoma patients, Aihara and associates found mammaglobin transcripts by RT–PCR in all histologically proven metastatic lymph nodes and in 31% of histologically negative lymph nodes [90]. All their control lymph nodes were negative by mammaglobin RT–PCR [90]. Zach and colleagues were able to detect mammaglobin mRNA in the PB of 28% of breast carcinoma patients of various stages, 5% of patients with non-breast carcinoma malignancies and in none of 27 healthy volunteers [86]. Two groups have recently used immunomagnetic-based assays for the detection of CTC in mammary carcinoma [87–91]. In one instance, the immunomagnetic procedure was followed by the assessment of telomerase activity by PCR [87]. This latter technique revealed telomerase activity in 21 of 25 (84%) metastatic breast carcinoma patients while all 9 healthy volunteers were telomerase negative. In order to detect CTC in breast or ovarian cancer, Engel and colleagues used immunobead isolation of tumour cells followed by fluorescence *in situ* hybridisation (FISH) for numerical chromosomal aberrations and immunocytochemistry for cytokeratins [91]. Circulating tumour cells from patients with localised tumour had a significantly lower percentage of chromosomal aberrations compared with those with relapsed disease and solid metastases [91]. Additional studies are needed to confirm the clinical value and specificity of mammaglobin RT–PCR and the latter two immunobead-based methods.

Table 5
Detection of circulating tumour cells in the peripheral blood of patients with cutaneous malignant melanoma using RT–PCR

	No. of RT–PCR positive patients/total no. of patients tested (%) according to AJCC stage		
Author [Ref.]	*n* (%) I–II	*n* (%) III	*n* (%) IV
Brossart [96]	1/10 (10)	6/17 (35)	29/29 (100)
Hoon [94][a]	13/17 (76)	31/36 (86)	63/66 (95)
Battayani [92]	2/10 (20)	22/51 (43)	16/32 (50)
Foss [97]	–	–	0/6
Pittman [98]	–	–	3/24 (13)
Kunter [95]	0/16	0/16	9/34 (26)
Mellado [99]	8/44 (18)	2/13 (15)	–
Curry [100][b]	48/160 (30)	60/116 (52)	–
Farthman [101]	6/46 (13)	7/41 (17)	16/36 (44)
Cheung [102][c]	5/17 (29)	4/54 (7)	4/27 (15)
Schitteck [103][b]	28/119 (24)	14/48 (29)	30/58 (52)

RT–PCR, reverse transcriptase–polymerase chain reaction; AJCC, American Joint Committee on Cancer; AJCC stage I, primary tumour $\leqslant$1.5 mm in thickness with no metastases; AJCC stage II, primary tumour $>$1.5 mm in thickness with no metastases; AJCC stage III, regional lymph node metastases; AJCC stage IV, distant metastases.

[a] In this study, the peripheral blood was analysed for four markers (*tyrosinase*, *p97*, *Muc 18*, *MAGE-3*).

[b] In both reports, the samples were tested for *tyrosinase* and *MART-1*. In all three articles [94,100,103], RT–PCR positivity was defined as positivity for any of the markers.

[c] *GAGE* mRNA alone was used as a marker in this article.

In all the other studies listed, *tyrosinase* alone was used as a marker for melanoma cells.

3.3. Malignant melanoma

The main current criteria to assess prognosis in malignant melanoma are the histopathological features of the primary tumour and the clinical presentation. However, these factors are of limited value in the advanced stages of the disease [92]. There is therefore a need for a better prognostic marker in these patients. The molecular detection of CTC and BM micrometastases has the potential of predicting outcome in patients with malignant melanoma. Smith and associates were the first to propose that melanoma cells could be detected in the PB using RT–PCR for *tyrosinase* mRNA [32]. Tyrosinase is a key enzyme in melanin biosynthesis that catalyses the conversion of tyrosine to dopa and of dopa to dopaquinone. This test is presumed to detect circulating melanoma cells since tyrosinase is one of the most specific markers of melanocytic differentiation [93] and melanocytes are not known to circulate. Furthermore most studies show that *tyrosinase* mRNA is not present in the PB of healthy individuals 34,92,94–96]. Since the original study of Smith and associates, several groups have attempted the detection of CTC in malignant melanoma using *tyrosinase* mRNA [34,92,94–101]. As shown in Table 5, the PCR positivity rates are extremely variable ranging from 0 to 100%. There is a correlation between the blood *tyrosinase* RT–PCR results and stage in some, but not all of the studies. These differing results could be in part explained by differences in the RNA extraction and PCR methodology [93]. These disparate findings could also be due to unrecognised contamination leading to false-positive results [93]. Indeed, Foss and colleagues acknowledged the presence of significant technical problems due to carry-over contamination that took 1 year to overcome [97]. 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 [34,92,95,99–101] (Table 6). We were also able to specifically detect *tyrosinase* transcripts in the BM of patients with American Joint Committee on Cancer

Table 6
Molecular prognosis in melanoma using RT–PCR[a]

Author [Ref.]	AJCC Stage	Marker	Sample	Endpoint	*P* value
Mellado [99]	I–III	*Tyrosinase*	Blood	DFS	0.003
		Tyrosinase	Blood	OS	0.001
Kunter [95]	IV	*Tyrosinase*	Blood	OS	$\leqslant$0.0006
Ghossein [34]	II	*Tyrosinase*	Blood	OS	0.01
	II	*Tyrosinase*	BM	OS	0.06
	III	*Tyrosinase*	Blood	OS	0.02
Curry [100]	I–III	*Tyrosinase*/*Mart* 1	Blood	DFS	0.0022
Cheung [102]	II–IV	*GAGE*	Blood/BM	OS	0.01
	III	*GAGE*	Blood/BM	OS	0.01
Shivers [106]	I–II	*Tyrosinase*	SLN	DFS	0.006
			SLN	OS	0.02

RT–PCR, reverse transcriptase polymerase chain reaction; AJCC, American Joint Committee on Cancer; AJCC stage (for description of stages see Table 5). OS, overall survival; DFS, disease-free survival; BM, bone marrow; SLN, sentinel lymph node.

[a] The relative risk was not available in all the above references. However, in each article RT–PCR positivity correlated with poorer survival time in a statistically significant manner except for Stage II BM testing [34]. Only those articles using Kaplan–Meier survival analysis are included in this table. In Curry's article, the samples were tested for *tyrosinase* and *Mart* 1. RT–PCR positivity was defined as positivity for any of the markers. In Cheung's article, RT–PCR positivity was defined as positivity for blood and/or BM.

(AJCC) stage II–IV melanoma [34]. In patients with AJCC stage III melanoma who were rendered surgically free of disease, we found that blood and/or BM positivity for *tyrosinase* mRNA was an independent predictor of poorer overall and disease-free survival [104]. In an effort to improve the clinical value of RT–PCR for *tyrosinase* mRNA, Brossart and colleagues developed a semi-quantitative RT–PCR assay [107]. According to these authors, the amount of *tyrosinase* transcripts increases with tumour burden in patients with metastatic diseases and decreases in patients responding to immunotherapy.

In order to increase our PCR positivity rate (only 19% *tyrosinase* positivity in blood and/or BM in advanced melanoma), we had to detect those occult melanoma cells that do not express *tyrosinase* mRNA. For that purpose, we used an additional marker termed *GAGE*. *GAGE* was identified in a human melanoma cell line [108] and belongs to a family of genes coding for an antigen recognised by autologous cytotoxic T lymphocytes. *GAGE* gene expression was identified by RT–PCR in a variety of tumour types including melanoma, sarcoma, neuroblastoma and non-small cell lung carcinoma [105,108]. It is silent in normal tissues except for the adult testis. Our group was able to detect *GAGE* mRNA in the PB and BM of melanoma patients [102], including some patients who were negative for *tyrosinase* mRNA, enhancing the sensitivity of our RT–PCR detection system to 45% in PB and/or BM (data not shown). *MART1*/*Melan* A is a melanocytic tissue-specific marker recognised by cytolytic T lymphocytes and detected by RT–PCR in the PB of melanoma patients [100,103]. In a recent article, Curry and colleagues reported a difference in metastatic potential between CTC that were RT–PCR-positive for *MART-1*/ *Melan*-A only and those positive for *tyrosinase* alone [109]. Patients with disseminated melanoma had a significantly lower incidence of *MART*-1 RT PCR-positive CTC (16%) than of *tyrosinase*-positive CTC (63%) [109]. It was suggested that the lack of expression of *MART-1*/*Melan* A in CTC of patients with recurrent disseminated tumour is due to the higher immunogenicity of *MART-1*/*Melan* A compared with tyrosinase [109]. Recently, a new marker termed melanoma inhibitory activity (*MIA*) was used for RT–PCR detection of circulating melanoma cells [110]. MIA is a malignant melanoma-derived growth regulatory protein highly expressed in melanomas but found at extremely low levels in keratinocytes, fibroblasts and lymphocytes when a standard single round RT–PCR is used [110]. Muhlbauer and associates found an increase in RT–PCR positivity with tumour burden in patients with metastatic disease and a decrease after adjuvant therapy [110].

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 [111]. 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 [111]. However, because of the rate-limiting step related to the number of sections that need to be examined, these techniques have not been widely used. To circumvent these problems, Wang and associates attempted the detection of lymph node micrometastases using RT–PCR for *tyrosinase* mRNA and showed this technique to be more sensitive than immunohistochemistry, or morphology [111]. 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 to 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 those with histologically positive sentinel lymph nodes and 65% of the histologically negative specimens [112]. Unfortunately, two of 18 control nodes without melanoma were *tyrosinase* PCR-positive [112]. We are currently following these patients in order to assess the prognostic value of this assay. Recently, Shivers and colleagues reported that the probability of recurrence and overall survival is influenced by the RT–PCR detection of *tyrosinase* mRNA in sentinel lymph nodes. These authors found a statistically significant difference in overall and disease-free survival between patients with RT–PCR-negative histologically negative lymph nodes and those with RT–PCR-positive histologically negative specimens [106]. These authors did not report on their RT–PCR results in control subjects without melanoma [106].

RT–PCR assays for the detection of CTC and micrometastases in melanoma seem very promising in view of: (1) the correlation between the RT–PCR assay results (especially blood tyrosinase) and outcome; and (2) the absence of accurate conventional prognostic marker in advanced melanoma. In order to clearly define the clinical usefulness of RT–PCR for occult melanoma cells,

methodological issues must be addressed using inter-laboratory studies [113].

3.4. Lung carcinomas

The 5-year survival rate of stage I–II non-small cell lung carcinoma is 30–50% after surgical resection. There is therefore a need to detect those patients with occult tumour cells who will recur and die. In order to better stratify patients with lung carcinomas, several groups have developed RT–PCR assays for *cytokeratin 19*, *CEA*, *Muc-1* and surfactant protein gene transcripts [83,114–118]. These assays were used to detect CTC and lymph node micrometastases. In one study, the CTC were semi-quantified by taking the ratio of *cytokeratin 19* band intensity from the second round of nested RT–PCR to the band intensity of a housekeeping gene (i.e. a widely expressed gene) after one round of PCR amplification [117]. In that article, serial measurement of the relative number of circulating cancer cells correlated with tumour burden and treatment response in non-small, as well as small cell lung carcinomas. The relationship between CTC and therapy response was however analysed in only a few patients [117]. Unfortunately, all the abovementioned markers including the surfactant protein gene products were shown to be expressed in control samples without carcinoma using RT–PCR (Table 4 [118]). There is clearly a need to develop more specific assays for CTC and micrometastases in lung carcinomas.

3.5. Gastrointestinal carcinoma

As with other solid tumours, 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 [20,77,119–121]. *CEA* mRNA was detected by RT–PCR in lymph nodes and BM specimens that were negative by immunohistochemistry for CEA and cytokeratin [20,121]. In patients with tumour node-metastasis (TNM) stage II colorectal carcinomas (who have no lymph node metastases by histology), the detection of *CEA* mRNA in regional lymph nodes was shown to correlate with a poorer 5-year survival rate [121]. However, in some studies, *CEA* mRNA was detected by RT–PCR in lymph nodes, blood and BM samples from individuals without epithelial malignancies [82,122–124]. *Cytokeratin 20* mRNA was used as a marker for colorectal carcinoma cells in lymph nodes, BM and blood [124,125]. This marker was unfortunately detected by RT–PCR in 72% of blood samples and all BM specimens from healthy individuals [126].

4. Future trends

Because of the limitations of PCR (e.g. contamination of samples, inability to quantify tumour cells or assess the cells for markers of disease progression), it is now clear that other approaches are needed for the detection and molecular characterisation of occult tumour cells. In the past 2 years, we and others have used immunomagnetic separation technology as a means to improve the detection of CTC [27,127–129]. In this technique, the specimen is incubated with magnetic beads coated with antibodies directed against a specific tissue type (e.g. Ber-EP4 antibody directed against carcinomas). The tumour cells are then isolated using a powerful magnet. The magnetic fraction can be used for downstream RT–PCR, *in situ* hybridisation or immunocytochemical analysis (Fig. 4). The sample used for

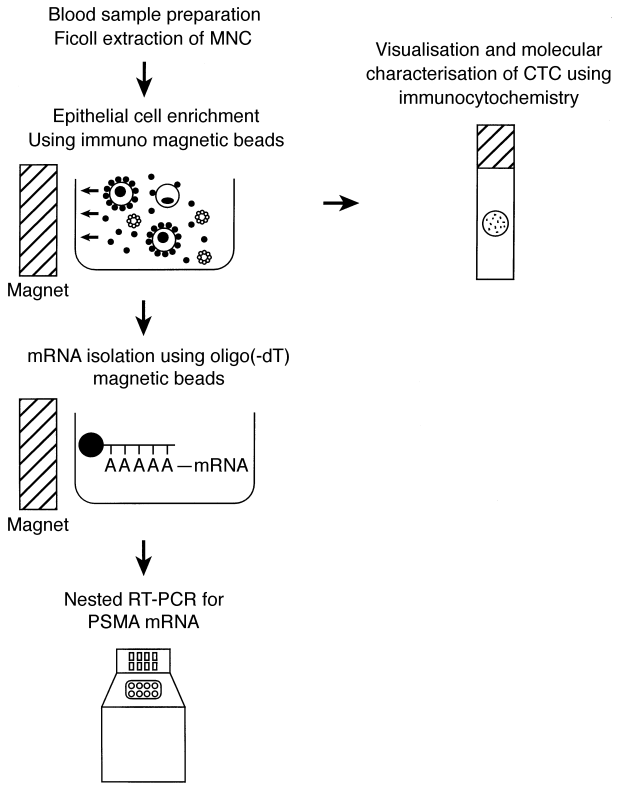

Fig. 4. Immunobead-based assay for the detection and molecular characterisation of circulating tumour cells (CTC). In this example, blood from a prostatic carcinoma patient is subjected to a Ficoll separation of nucleated cells . The mononuclear cell (MNC) layer is incubated with magnetic beads coated with the Ber-EP4 anti-epithelial cell antibody directed against carcinomas. The magnetic fraction is then isolated using a powerful magnet and is rich in tumour cells. The cells present in the magnetic fraction are then lysed and their mRNA isolated using oligo dT magnetic beads. This preparation is then ready for reverse transcriptase–polymerase chain reaction (RT–PCR) for prostate specific membrane antigen (*PSMA*) mRNA, a prostatic specific marker (left). The isolated magnetic cell fraction can also be cytospun on glass slides and subjected to immunofluorescence, immunoperoxidase and *in situ* hybridisation in order to characterise and quantify the CTC (right).

RT–PCR will therefore be considerably enriched in tumour cells with a minimal background of non-neoplastic cells. In addition, oligo (dT) coated magnetic beads can be utilised to isolate mRNA. The use of mRNA instead of total RNA as starting material decreases the background resulting from unspecific priming and helps to 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. Immunocytochemical analysis of the specimen will allow better quantification of the tumour cells, and their assessment for various markers of tumour proliferation and progression. This will help to monitor the effect of targeted therapy (e.g. the monoclonal antibody against HER-2, commercially known as Herceptin), better stratify patients with solid tumours and shed more light on the dynamic process of metastases. The management of patients with solid malignancies will therefore become more rational, economical and conservative.

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