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Original Paper

The Detection of Micrometastases in the Peripheral Blood and Bone Marrow of Patients with Breast Cancer Using Immunohistochemistry and Reverse Transcriptase Polymerase Chain Reaction for Keratin 19

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The aim of this study was to determine whether reverse transcriptase polymerase chain reaction (RT-PCR) for keratin 19 (K19) provides additional information when combined with immunohistochemistry when used to detect micrometastases in blood and bone marrow in patients with primary breast cancer. We studied 78 patients with breast cancer who had no evidence of distant metastases. We collected blood and bone marrow, separated the mononuclear fraction and carried out RT-PCR and immunohistochemistry for K19. RT-PCR was done by two 40-cycle rounds using nested primers. In initial experiments, RT-PCR was shown to be capable of detecting one tumour cell in one million normal bone marrow cells, which was at least 10 times more sensitive than immunohistochemistry, while retaining specificity. Five per cent of the peripheral blood and 22% of the bone marrow samples contained K19 positive cells by immunohistochemistry staining. Using RT-PCR, these proportions increased to 25% and 35%, respectively. This represents a significantly greater detection frequency ($P < 0.001$ and $P = 0.03$, respectively). RT-PCR for K19 is a more sensitive method for detecting micrometastases in patients with primary breast cancer when compared with immunohistochemistry. © **1997 Elsevier Science Ltd.**

Key words: micrometastases, breast cancer, keratin 19

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INTRODUCTION

IMMUNOHISTOCHEMICAL STAINING techniques have shown the presence of cancer cells in the bone marrow of approximately 20–30% of patients with operable breast cancer [1–4]. The occurrence of these micrometastases has been related to prognostic features of the primary, such as tumour size and the presence of vascular invasion, and lymph node involvement [5, 6]. In addition, the presence of bone marrow micrometastases has been strongly correlated to early recurrence and shorter overall survival [3, 6]. The risk increases with increasing number of cells detected in the bone marrow [3, 7].

However, up to 10% of patients relapse with no histological or immunohistochemical evidence of bone marrow micrometastases, following resection of the primary tumour [3, 8]. Additionally, only a proportion of patients remain positive for micrometastases following primary surgery [8]. Detection of metastatic cells in peripheral blood was rare [1, 4].

The sensitivity of immunohistochemical methods using monoclonal antibodies to epithelial antigens or cytokeratins has been estimated at approximately one cancer cell per

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10^4–10^5 normal bone marrow cells [9, 10]. Measurement of a tissue-specific gene transcript following PCR amplification, while retaining specificity, has been reported to increase the sensitivity by up to 100 times [11], with detection of a single neuroblastoma cell in 10^7 peripheral blood mononuclear cells.

We have shown that measurement of PCR amplified K19 is much superior to immunohistochemical techniques as a means of determining the presence of micrometastases in the axillary lymph nodes of patients with breast cancer [12]. However, practical considerations prevent the repeated sampling of lymph node tissue. A similar technique applied to peripheral blood or bone marrow would offer significant advantages, allowing a better assessment of the prognosis of patients with breast cancer and monitoring of the response to adjuvant chemotherapy.

In this study, we have used RT-PCR assay [12], to detect K19 mRNA transcripts in samples of peripheral blood and bone marrow from breast cancer patients. We have also analysed samples using the immunohistochemical staining technique, with an antikeratin 19 antibody, to compare these two methods.

PATIENTS AND METHODS

Chemicals

MMLV reverse transcriptase was obtained from GIBCO BRL (Paisley, U.K.) and Taq polymerase from Penninsula Laboratories (U.K.). Random hexamers and dNTPs from Pharmacia (Uppsala, Sweden) and ^{32}P dCTP (3000 Ci/mmol) was from Amersham International, U.K. RNAzol was from Biogenesis (Bournemouth, U.K.). All other reagents were obtained from Sigma (Dorset, U.K.) unless indicated.

Cell lines

MCF7 human breast cancer cells were maintained in monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin. When required, cells were harvested by trypsinisation.

Sensitivity assay

Monolayer cultures of MCF7 cells were harvested with trypsin and, following centrifugation, washed by resuspension in PBS (phosphate-buffered saline) and then disaggregated by passing through a 26-gauge needle. Cells were counted using a haemocytometer, and serial dilutions of the cell suspension prepared. Suitable volumes of media containing 5–500 MCF7 cells were mixed with 5×10^6 normal bone marrow cells after cell separation to give a ratio of MCF7 cells to bone marrow cells of $1{:}10^6$, $1{:}10^5$ and $1{:}10^4$. These preparations were then used either to prepare smears for immunohistochemistry or for RNA extraction.

Patients

Peripheral blood and bone marrow specimens were collected from 113 patients at Charing Cross and St. George's Hospitals in London, U.K. and the University Clinic in Frankfurt, Germany. Clinical and pathological details for 88 patients are given in Tables 1 and 2. All patients gave their informed consent to the study, which had Ethics Committee approval.

Table 1. Clinical data on patients with breast cancer [84] *and ductal carcinoma in situ* [4]

	Number of patients
Total	88
Age (years)	
Range	31–85
Mean	58.2
Menopausal status	
Pre-	14
Peri- and post-	74
Operation	
Mastectomy	27
Wide local excision	59
Wire localisation	2

There were 75 patients in this study who had untreated primary breast cancer with no evidence of distant metastatic disease by pre-operative staging. The staging included serum calcium, alkaline phosphatase and liver function tests, full blood count and a chest X-ray, liver ultrasound, isotope bone scan and skeletal survey.

In addition, there were 4 patients with ductal carcinoma *in situ*, 4 patients who had a breast carcinoma excised previously and had no evidence of recurrent disease, and 5 patients who had metastatic carcinoma of the breast with radiological evidence of bone involvement.

Table 2. Pathological data on patients with breast cancer

Parameter	Patient number
Tumour type	
Invasive ductal carcinoma	73
Invasive lobular carcinoma	8
Tubular	2
Medullary	1
Ductal carcinoma *in situ*	4
Tumour size (cm)	
0–1	16
1.1–2	31
2.1–5	29
5.1+	5
Unknown	7
Tumour grade (Bloom and Richardson)	
Well differentiated	13
Moderately differentiated	45
Poorly differentiated	18
Unknown	12
Vascular invasion	
Present	20
Absent	17
Unknown	51
Oestrogen receptor status	
Positive	47
Negative	22
Unknown	19
Progesterone receptor status	
Positive	32
Negative	14
Unknown	42
Axillary node involvement	
None	49
1–3	14
4 or more	20
Unknown	5

For negative controls, peripheral blood and bone marrow was obtained from 25 patients undergoing a diagnostic or follow-up marrow aspirate in the haematology clinic and known not to have an epithelial malignancy.

Specimen collection

The specimens were collected from patients under general anaesthetic just before their breast operation. Under sterile conditions, a bone marrow aspirate was taken from each posterior iliac crest using a 16 Saleh needle. To minimise the risk of skin contamination, an incision was made in the skin with a scalpel before introducing the needle. Approximately 10 ml of bone marrow and venous blood was aspirated from each site in a syringe containing 1 ml of 6% ethylenediaminetetraacetic acid (EDTA) as anticoagulant and then pooled.

In addition, 20 ml of peripheral blood were taken from a vein in the antecubital fossa and mixed with 1 ml of 6% EDTA. White blood cell counts were determined using a Coulter counter STKR.

Cell separation

The peripheral blood and bone marrow were processed separately under sterile conditions using the same procedure. Each sample was gently overlaid on to an equal volume of Lymphoprep (Nyegaard, Oslo, Norway), and centrifuged for 20 min at 500*g* at room temperature. The nucleated cells, including the metastatic cancer cells, collected at the visible interface between the Lymphoprep and the serum [13].

The serum supernatant and interface were removed, diluted to a total volume of 50 ml in RPMI medium, and centrifuged for 6 minutes at 350*g* at room temperature. The resulting cell pellet was gently resuspended in fresh RPMI medium and again centrifuged for 6 min at 350*g* at room temperature. All but 2 ml of the supernatant was then removed and the cell pellet was resuspended in this, and an aliquot removed for cell counting using the Coulter counter. The sample was equally divided for subsequent analysis by either anticytokeratin 19 immunohistochemistry or RNA extraction and measurement of K19 mRNA expression.

For immunohistochemistry, the sample was centrifuged for 5 min and the cell pellet resuspended in a small volume (approximately 100 μl) of the supernatant. Aliquots of this suspension (50 μl) were smeared on to two frosteal slides to form a monolayer of cells. The smears were air-dried, fixed in 100% ethanol for 1 h and then air-dried for 30 min before storage at −20°C.

The sample for RNA extraction was centrifuged for 5 min and all the supernatant discarded. The cell pellet was snap-frozen in liquid nitrogen and stored at −70°C.

Staining procedure for immunohistochemistry

The smears were equilibrated to room temperature and allowed to dry. To block endogenous alkaline phosphatase activity, slides were placed in 20% glacial acetic acid for 10 min, washed in water and then in 2% periodic acid for 10 min. The slides were rinsed sequentially in tap water and PBS, before incubation in a humidified chamber for 20 min with PBS buffer containing a 5% bovine serum albumin and 5% goat serum. Excess fluid was wiped off the slide and the primary antibody, a mouse anticytokeratin 19 antibody (4.62, Sigma, U.K.), diluted to 5 μg/ml was added to each slide and incubation carried out at room temperature for 90 min.

The slides were washed in PBS and then incubated with an alkaline phosphatase conjugated goat antimouse antibody (S3721, Promega, U.K.), which had been diluted to 1:250 in PBS/bovine serum albumin buffer containing 10% human serum. Following washing, the substrate solution, NBT/BCIP (Vector Laboratories, U.S.A.), was applied to each slide and smears then incubated for 30 min. The smears were dehydrated in graded alcohol and counterstained with Gill's haematoxylin, before mounting using Histomount (National Diagnostics, U.K.). Each slide was screened under low-power light microscopy for positive cells and the morphology of each positive cell was confirmed under high power. A smear was considered to be positive if one or more stained epithelial cancer cell was observed.

Oligonucleotide primers

The primers for K19 and GAPDH were as previously described [12] and synthesised using phosphoramidite chemistry on an Applied Biosystems DNA Synthesizer. Both sets of K19 primers spanned at least one intron and sequences were designed to maximise differences with the K19 pseudogene [14–16].

RNA extraction

Total cellular RNA was extracted from the frozen peripheral blood and bone marrow samples using the acid–guanidium–phenol–chloroform technique [17] utilising RNAzol. Following extraction, the integrity of the RNA was checked electrophoretically and quantified spectrophotometrically, and samples diluted to approximately 0.3 μg/μl in water and stored at −70°C.

Reverse transcription and PCR amplification

This was carried out exactly as described previously [12] and two 40 cycle rounds of amplification were performed. In brief, 4 μg RNA was reverse transcribed in a total reaction volume of 20 μl, and 4 μl was added to the PCR mix (100 μl) for the first amplification using the primers already described [12]. Subsequently, 1 μl of the first reaction was carried over for the second amplification (in a 50 μl reaction mix) using the nested primers K6 and K7 described previously [14]. Control reactions were performed in parallel and involved using product from mock RT reactions which contained RNA but no enzyme.

The conditions for amplification using the two pairs of primers were optimised. The amount of cDNA added to the PCR reaction and the number of amplification cycles performed to maximise the yield of product were established. Different combinations of primers at different annealing temperatures were tried and different concentrations of magnesium chloride were tested.

The positive controls were normal bone marrow or peripheral blood cells spiked with MCF7 cells. 'No DNA' reagent controls, containing the necessary components for PCR except template DNA, and negative controls were also included with every amplification.

Gel electrophoresis

Aliquots of chloroform-extracted PCR (20 μl) were electrophoresed at 150 V for 2 h in Tris–acetate EDTA buffer on a 1.5% agarose gel containing ethidium bromide. HaeIII

digested OX 174 DNA markers or the CAMBIO DNA ladder were run simultaneously. After two rounds of amplification, samples which had a discernable band corresponding to 319 bp on examination of the gel under ultra-violet light were considered as positive. The RT-PCR product was indentified by the band on gel electrophoresis and confirmed by sequencing.

RESULTS

Cell separation

Density gradient centrifugation resulted in an average yield of 20.4% (range 0.8–80%) for peripheral blood, and 28.2% (range 2.1–82%) for bone marrow, of the total initial number of white blood cells. The average number of cells examined was 23.0×10^6 (range $1.5–104 \times 10^6$) for peripheral blood and 149.2×10^6 (range $3.6–620 \times 10^6$) for bone marrow, and each sample was equally divided for immunohistochemical and RNA analysis.

Specificity and sensitivity of immunohistochemistry and RT-PCR for detection of K19 expression

Initial experiments were performed to determine whether K19 expressions could be detected in samples from patients with no evidence of breast or other epithelial malignancy. We examined 10 paired samples of bone marrow and peripheral blood and a further 15 peripheral blood samples from such a group of unselected patients. Using immunohistochemical staining with an anticytokeratin 19 antibody, we were unable to detect any K19 positive cells. RT-PCR, using primers to amplify K19 mRNA, on RNA extracted from aliquots of the same samples was also performed. No signal corresponding to K19 mRNA was detected following two rounds of 40 cycles of amplification by ethidium staining of electrophoresed material. Simultaneously amplified products (of 379 bp) corresponding to a housekeeping gene, *GAPDH*, were clearly seen in all samples after the first PCR, confirming the presence of amplifiable cDNA and ensuring that the absence of K19 product was not due to the lack of input RNA (data not shown).

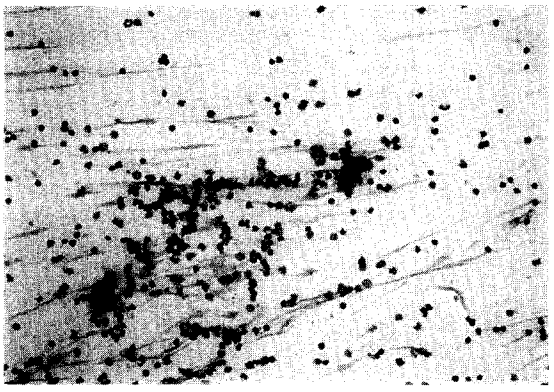

Figure 1. Immunostaining of a smear of normal bone marrow cells spiked with MCF7 cells with an antikeratin 19 antibody. Examination by light microscopy (magnification 100×) showed cytoplasmic staining reactivity only in epithelial MCF7 cells.

We assessed the sensitivity of both the immunohistochemical and PCR techniques by preparing smears or RNA from 5×10^6 normal bone marrow cells to which were added 5–500 MCF7 cancer cells. We were able to detect K19 immunopositive cells at a dilution of one MCF7 cell in 10^5 bone marrow cells on 6/7 separate occasions, but not at higher dilutions. Increasing numbers of immunopositive cells were detected as more MCF7 cells were added. Approximately 20–30% of the MCF7 cells added to the separated normal bone marrow cells were counted on the stained smears at all dilutions. The cytoplasmic staining appeared specifically on epithelial cells, staining over 95% of MCF7 cells, and there was no cross-reactivity with other cell types in the blood or bone marrow smears (Figure 1).

After two rounds of PCR amplification of RNA from the various cell preparations, a 319 bp product indicating amplification of K19 mRNA was visualised by ethidium staining at a dilution of one MCF7 cell in 10^6 bone marrow

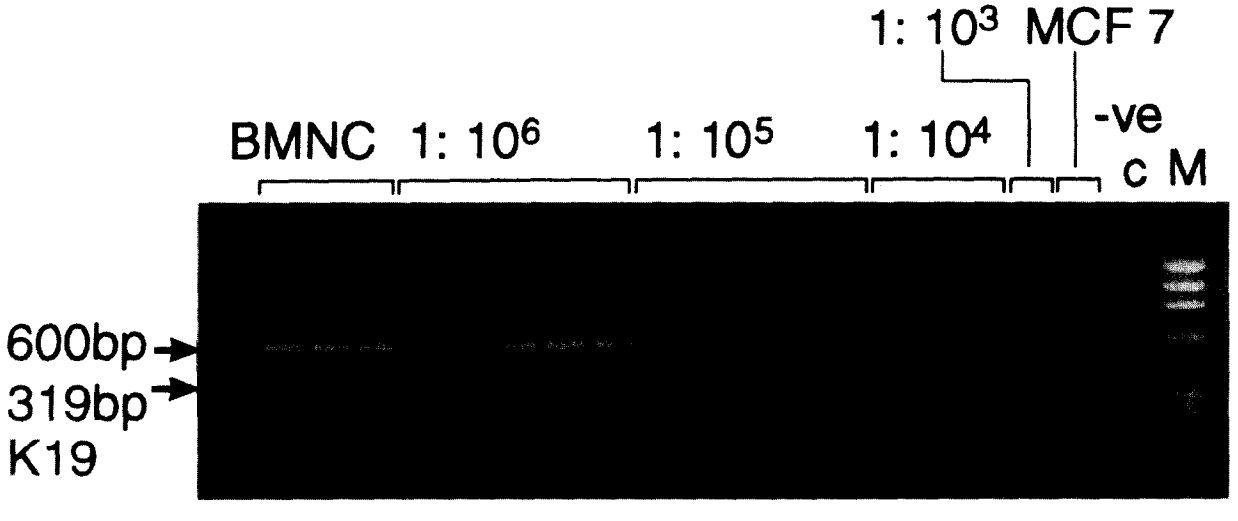

Figure 2. Sensitivity of detection of K19 PCR product in normal bone marrow samples spiked with MCF7 cells in the proportions indicated. RT product from MCF7 cells, normal bone marrow cells untreated (BMNC) or spiked with MCF7 cells was amplified by two 40-cycle reactions using K19 primers K1 and K2 and then K7 and K6. PCR product was electrophoresed through 1% agarose and stained with ethidium bromide. The mRNA-derived 319 bp product is indicated, and was easily distinguishable from the genomic DNA-derived 600 bp product. Lane -ve c is the negative control, and HaeIII digested OX 174 DNA markers are shown in lane M.

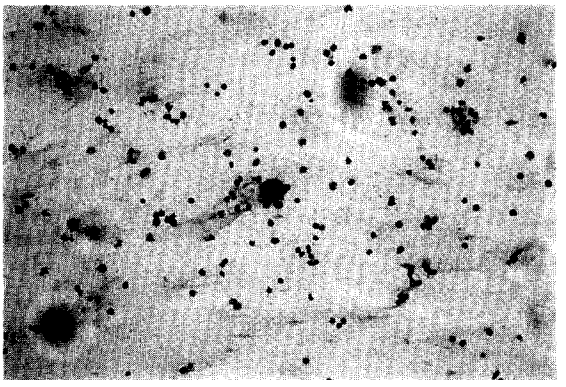

Figure 3. Antikeratin 19 immunostaining of the bone marrow of a patient with breast cancer showing a positive tumour cell. Magnification 100×.

cells on 4/5 separate determinations (Figure 2). The staining intensity of the nested product band at 319 bp increased as expected in the preparations containing higher numbers of MCF7 cells. In most samples, a band corresponding to an approximately 600 bp product was also observed. We have previously shown this to be derived from contaminating DNA [12]. The intensity of this band decreased as that of the 319 bp band increased with increasing numbers of MCF7 cells. This may be the result of preferential amplification of the mRNA template due to the smaller size of the PCR product compared to the genomic DNA template.

Detection of breast cancer cells in peripheral blood and bone marrow

In the samples from the 75 patients with primary invasive tumours and no evidence of distant metastatic disease on conventional staging, we found, by a combination of the two methods, K19 positivity in 20/75 (27%) peripheral blood and 27/65 (42%) bone marrow samples. By immunohistochemistry alone (Figure 3), we detected K19 positive cells in 4/75 (5%) peripheral blood and 14/65 (22%) bone marrow samples. The number of cells identified in each case ranged from 1 to 10 (mean 4 cells) in peripheral blood and 1 to 40 (mean 7.5 cells) in bone marrow. By RT-PCR (Figure 4) we observed K19 expression in 19/75 (25%) peripheral blood and 23/65 (35%) bone marrow samples. These were significantly greater percentages than those detected by immunocytochemistry ($P < 0.001$ and $P = 0.03$, respectively). Further analyses are presented in Table 3. Thus, in the peripheral blood, only 4/20 (20%) positives were detected by immunohistochemistry and 19/20 (95%) by RT-PCR. In the bone marrow, 14/27 (52%) were detected by immunohistochemistry and 23/27 (85%) by RT-PCR. If positivity was considered in either peripheral blood or bone marrow, we detected 17 of the 36 positives (47%) by immunohistochemistry and 34/36 (94%) by RT-PCR. There was no correlation between K19 detection (either by immunochemistry or PCR) and pathological size of the primary tumour, presence or absence of vascular invasion, or node involvement. Nor was there any relationship with steroid receptor content.

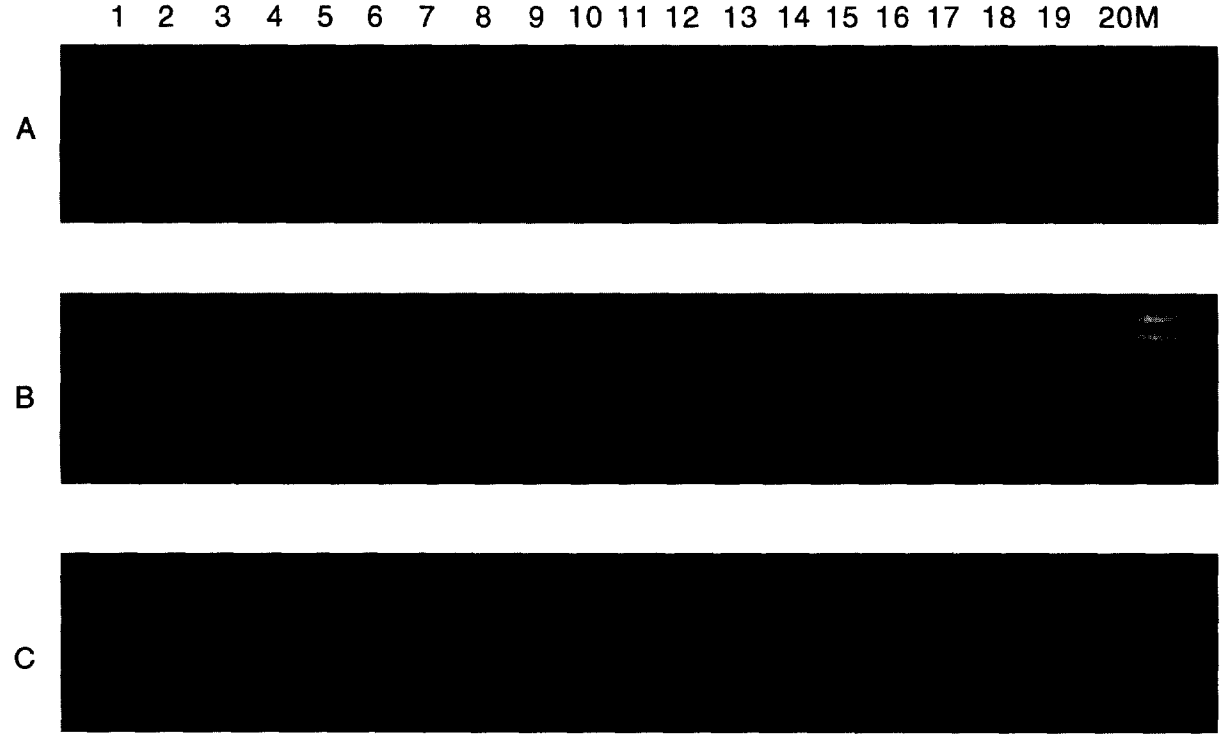

Figure 4. RNA was extracted from 48 peripheral blood and bone marrow samples from patients with breast cancer and K19 amplified by two rounds of 40 cycles of RT-PCR. Aliquots (20 μl)of the product were analysed by electrophoresis on a 1% agarose gel and ethidium staining. A lower band at 319 bp indicates K19 mRNA expression in that sample (e.g. lanes A3, B6, C3). Lanes C9–C12 contained the negative controls, and lanes C11 and 12 were from patients without cancer. Lane C13 was run with the HaeIII digested OX 174 DNA markers.

Table 3. Comparison of detection rate of micrometastases in peripheral blood and bone marrow

	Peripheral blood	Bone marrow	Peripheral blood or bone marrow
Total no. patients	75	65	75
Negative by both IH and PCR	55	38	39
Positive by either IH or PCR	20	22	36
Positive by both IH and PCR	3 (15%)	10 (37%)	15 (42%)
Positive by IH only	1 (5%)*	4 (15%)†	2 (5%)
Positive by PCR only	16 (80%)	13 (48%))	19 (53%)

The percentages in parentheses indicate the proportion of K19 positive cells detected by that technique. The McNemar test was used to compare the two techniques.
* Significantly more patients detected by PCR ($P < 0.001$).
† Significantly more patients detected by PCR ($P = 0.03$).
IH, immunohistochemistry.

Overall, considering both the positive and the negative samples, the concordance between peripheral blood and bone marrow samples analysed by immunohistochemistry was 79% (58 negative + 1 positive with both tests, 59/75) compared with 66% by RT-PCR (35 negative + 8 positive with both tests, 43/65; Table 4). However, if only those cases that were positive in either peripheral blood or bone marrow are considered, the concordance for immunohistochemistry was low (6%); 82% (14/17) of the positive cases were found by analysis of the bone marrow, and only 24% (4/17) from the peripheral blood. Using RT-PCR there was a better rate of concordance between peripheral blood and bone marrow samples (27%); detection of positives was also more frequent in the bone marrow (23/30 77%) than the peripheral blood (15/30 50%).

We also examined peripheral blood and bone marrow from 4 patients with *in situ* carcinoma of the breast. These samples were negative by both techniques apart from one patient who had K19 positive cells in both blood and bone marrow. Of the group of 5 patients with radiological evidence of bone metastases, one had single K19 positive cell in the peripheral blood detected by immunohistochemical staining and 4 had positive cells in the bone marrow. By RT-PCR, we detected K19 bands in 4 of the 5 patients in both blood and bone marrow. One patient had no positive cells in blood or bone marrow by either technique.

A further 4 patients had had the primary tumour excised at least one year previously and had no recorded evidence of recurrent disease. In 2 patients, we detected positive cells in the peripheral blood by RT-PCR; in one of these patients immunohistochemistry confirmed the presence of tumour cells in the bone marrow.

Table 4. Comparison of immunohistochemistry and RT-PCR for the detection of micrometastases

	IH	RT-PCR
Total no. of patients with K19-positive cells in either blood or bone marrow	17/75	30/65
Positive in both blood and bone marrow	1 (6%)	8 (27%)
Positive in blood only	3 (18%)	7 (23%)
Positive in bone marrow only	13 (76%)	15 (50%)

A comparison of the two sampling sites, peripheral blood and bone marrow, in the detection of K19 positive cells by immunohistochemistry and RT-PCR. The proportion of K19-positive samples found at each site is expressed as a percentage in brackets of the total number of K19-positive patients detected by that technique.

DISCUSSION

In this study, we demonstrated that RT-PCR using K19 primers is a sensitive and specific technique for the detection of tumour cells in the peripheral blood and bone marrow of patients with breast cancer. This assay detected K19 expression from MCF7 cells serially diluted down to 1 MCF7 cell in 10^6 normal bone marrow cells. This level of sensitivity was at least 10 times greater than that achieved with immunohistochemistry and was comparable to that reported in other studies [18, 19]. RT-PCR may be capable of detecting 1 in 10^7 cells, depending on the efficiency of the primers, the abundance of the gene transcript and the design of the assay.

Detection of mRNA transcripts might be expected to be more sensitivity than immunohistochemistry as multiple copies of mRNA provide a greater target, and downstream modification and translation may reduce the expression of protein. Each target may then be amplified 10^6 times before detection. In contrast, the process of smearing and immunostaining resulted in the detection of only approximately 30% of the added MCF7 cells. A similar proportion was observed by Osborne and associates [9]. This may be attributed to the settling or clumping of the MCF7 cells and/or the loss of cells during smearing, and necessarily compromises the sensitivity of the immunohistochemical detection.

A further disadvantage of the immunohistochemical procedure is that a trained cytologist is required to confirm the identity of stained cells. Although our monoclonal antibody to K19 appeared specific, cross-reactivity of other antibodies with different cells has been described. Using antibodies to epithelial membrane antigen, occasional staining of plasma cells, early myeloid cells and degenerate cells can occur due to weak expression of this antigen by those cells [20].

The specificity of RT-PCR relies on the detection of a unique or overexpressed gene in the tumour cell. Certain malignancies are characterised by unique targets; in chronic myeloid leukaemia there is a specific bcr/abl chromosomal translocation that has been exploited [21], and prostate cancer cells overexpressed prostate-specific antigen [22, 23]. No unique markers have been identified in breast cancer cells. Although gene rearrangements on chromosome 17q have been described in some familial breast cancers, and point mutations in known oncogenes are frequently associated with sporadic breast cancer, no consistent genetic alteration has been found. We have, therefore, used an epithelial-specific marker, K19, as this has been reported to be present in most benign and malignant breast tissue [24], and we have found it to be expressed in all breast carcinomas examined with the exception of medullary carcinomas (A. Schoenfeld) but absent in normal haematological tissue [18, 25, 26]. This was confirmed in this study in that no K19 was detected in the peripheral blood or bone mar-

row of patients with benign breast disease or non-epithelial malignancies.

RT-PCR improved the detection rate, compared with immunohistochemistry, of occult metastases in the peripheral blood ($P < 0.001$) and bone marrow ($P = 0.03$) of patients with breast cancer. By immunohistochemical staining, we detected bone marrow micrometastases (22%) at a similar frequency to that of other studies (16–31%) [1–4]. The RT-PCR method increased the detection rate (35%) in bone marrow samples. In addition, RT-PCR was much more effective in the detection of K19-positive cells in peripheral blood. However, RT-PCR failed to detect K19 immunopositive cells in one peripheral blood and four bone marrow samples. The reasons for this are not clear. It may be that these immunohistochemically detected cells were not viable [27] or that they were 'dormant' with low metabolic activity, as defined by their inability to synthesise CK19 mRNA.

Both techniques detected more positives in bone marrow than peripheral blood. This may partly be a consequence of examining approximately seven times more cells for the bone marrow compared to the peripheral blood samples. It may also be that bone marrow acts as a filter for concentrating circulating breast cancer cells and this is reflected in the propensity of breast cancer cells to form overt bone metastases. A recent study of patients with lymphomas has also concluded that sampling bone marrow is more accurate than peripheral blood [28].

We conclude that immunohistochemical analysis detected only a minority of positive samples from the peripheral blood, and that sampling of bone marrow is essential for this technique to contribute useful data. RT-PCR similarly detected more positives in the bone marrow, but sampling of both the peripheral blood and bone marrow significantly improved the detection rate.

The concordance in the detection of K19 positive cells between blood and bone marrow samples by either technique was disappointingly low (immunohistochemistry (6%) and RT-PCR (27%)). A larger sample volume would probably increase the detection rate for both techniques, but may be impractical for repeated sampling. Mansi and colleagues [8] performed bone marrow sampling at eight different sites pre-operatively.

In patients with radiological evidence of bone involvement, only 1 patient had no positive cells by either immunohistochemistry or RT-PCR. This patient was receiving chemotherapy and was clinically in remission. The others had K19-positive cells in the bone marrow by immunohistochemical staining, and 1 patient had a single positive cell in the peripheral blood. These samples were positive in both blood and bone marrow by RT-PCR.

Somewhat surprisingly, one patient with ductal carcinoma *in situ* also had a positive result by both techniques. This patient had a wide local excision performed with local radiotherapy to the breast, and histological examination reported micro-invasion associated with the *in situ* ductal carcinoma.

Our study has directly compared immunohistochemistry and RT-PCR as well as the comparative merits of sampling peripheral blood and bone marrow. The study was designed to utilise a small volume of bone marrow and peripheral blood to improve the acceptability to patients and facilitate the analysis. Increasing the number of sites of bone marrow aspiration [2] or increasing the volume of peripheral blood for analysis may increase the detection of patients with occult disease up to a point and the increased sensitivity of RT-PCR will reduce the sampling error and workload. The definite answer to the prognostic significance of these RT-PCR detected micrometastases will require extended follow-up to assess the recurrence rate and survival of this cohort of patients. Further patients have been recruited into this study and technical improvements have been developed (unpublished results) to quantify the RT-PCR assay and simplify the routine processing of large numbers of patients.

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