Sensitive fluorescent in situ hybridisation method for the characterisation of breast cancer cells in bone marrow aspirates

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Abstract

***Aim*—The presence of malignant cells in the blood and bone marrow of patients with cancer at the time of surgery may be indicative of early relapse. In addition to their numbers, the phenotypes of the micrometastatic cells might be essential in determining whether overt metastases will develop. This study aimed to establish a sensitive method for the detection and characterisation of malignant cells present in bone marrow.**

***Methods*—In spiking experiments, SKBR3 cells were mixed with mononuclear cells in known proportions to mimic bone marrow samples with micrometastatic cells. Tumour cells were extracted using SAM-M450 Dynabeads coupled to the MOC-31 anti-epithelial antibody, and were further analysed for amplification of erbB2 and int2 by fluorescent in situ hybridisation (FISH). erbB2 and int2 copy numbers were also determined in 15 primary breast cancers, and bone marrow samples from patients with amplification were analysed for micrometastatic cells by immunomagnetic enrichment and FISH.**

***Results*—In model experiments, cells with amplification could be detected in bead selected fractions when ratios of tumour cells (SKBR3) to mononuclear cells were as low as 10:10^7. Among the tumour samples, eight showed increased copy numbers of erbB2 and/or int2, and three of these patients had detectable numbers of tumour cells in their bone marrow: 4000, 540, and 26 tumour cells/10^7 mononuclear cells, respectively. The patient with 540 tumour cells/10^7 mononuclear cells showed high level amplification of erbB2 and suffered from a particularly aggressive disease, whereas the patient with 4000 tumour cells/10^7 mononuclear cells had favourable disease progression.**

***Conclusion*—These results demonstrate the feasibility and advantage of combining immunomagnetic selection and FISH characterisation of cancer cells in bone marrow samples. It is possible that molecular characterisation of such cells could provide prognostically valuable information.**

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Keywords: fluorescent in situ hybridisation; erbB2; int2; breast cancer; bone marrow aspirates

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In primary breast cancer, the number of axillary nodes with tumour cell infiltration is at present the strongest predictor of clinical outcome.[1] However, a substantial number of patients with negative lymph nodes at the time of surgery develop metastatic disease,[2] and additional parameters to identify women at high risk are needed.

Several studies have indicated that the presence of tumour cells in the bone marrow of patients with breast cancer is associated with a shorter overall survival, regardless of lymph node status.[3 4] Moreover, Diel *et al* have shown recently that this parameter is the most powerful predictor of outcome in patients with tumours less than 2 cm in diameter.[5] Thus, the detection of circulating tumour cells in the bone marrow (and possibly in the blood) might provide important information for the prediction of disease progression. However, the value and usefulness of studying such associations as a possible aid in therapeutic decisions depend on the availability of practical and sensitive detection methods.

Immunocytochemical and molecular assays have been developed to study the presence of breast carcinoma cells disseminated to the bone marrow, blood, or lymph nodes.[6 7] For similar purposes, we have established a rapid and simple immunomagnetic procedure with improved sensitivity compared with immunocytochemistry.[8] The method can be used on fresh or frozen cell fractions, and allows quantitation of the tumour cells by counting the number of bead rosetting cells under light microscopy.[9]

It has been suggested that the number of micrometastatic cells in the bone marrow could reflect the peripheral tumour burden in each patient.[3] However, it might be the properties of the malignant cells in the bone marrow, rather than their numbers, that determine the clinical outcome. Therefore, it is of interest to characterise such cells for markers associated with an aggressive tumour phenotype. In most cases studied, relatively few tumour cells were found in the clinical samples,[6 10] and the numbers of tumour cells available for further analysis were very limited, requiring a sensitive method for their enrichment and characterisation.

One possibility is to use fluorescent in situ hybridisation (FISH) for detecting amplifications and other genetic alterations in tumour cells. The sensitivity of the method is high, and clonal variation among the cancer cells can be revealed. Because FISH depends on molecular marker probes that give clear cut qualitative

results, only a small number of cells are needed for analysis.

In breast cancer, amplification of the erbB2 and int2 proto-oncogenes is relatively frequent, being found in 25–30% and 15% of cases, respectively.[11–15] Notably, erbB2 amplification and overexpression, as well as int2 amplification, have been associated with poor prognosis.[11 14–22] Therefore, these markers would appear to be good candidates for FISH studies on circulating malignant cells, as possible prognostic indicators. We established a sensitive technique for the detection and amplification analysis of tumour cells in bone marrow by combining immunomagnetic enrichment and FISH.

Materials and methods

TUMOUR MATERIAL

Tumour cells and culture media

The epithelial cell line SKBR3, originally established from a pleural effusion of a breast adenocarcinoma, was obtained from the American Tissue Culture Collection (Rockville, Maryland, USA). The cell line was maintained as a monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 10^{-8} M insulin, 10^{-8} M hydrocortisone, and 10^{-8} M β-oestradiol (all from Sigma, St Louis, Missouri, USA).

In spiking experiments, SKBR3 cells were mixed with 10^7 mononuclear cells to give 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} tumour cells to mononuclear cells, thereby mimicking bone marrow samples with micrometastases.

Primary tumour material

Primary tumour material was obtained directly after surgery from 15 patients with advanced breast carcinoma. The tissues were cut into small pieces, frozen in liquid nitrogen, and stored at −70°C.

Bone marrow cells

Bone marrow samples were aspirated from the iliac crest of patients at the time of surgery. After Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation (1000 ×*g* for 10 minutes), mononuclear cells from the interface layer were washed in phosphate buffered saline with 1% human serum albumin (PBS + 1% HSA; Octapharma AG, Ziegelbrucke, Switzerland), resuspended, counted, and frozen.

For immunomagnetic detection experiments, the frozen bone marrow cells were thawed and resuspended in PBS + 1% HSA, washed once by centrifugation, and resuspended in PBS + 1% HSA. The mononuclear cells were counted by adding 0.01% crystal violet stain (Merck, Darmstadt, Germany) and diluting the cells to a final concentration of 10^7 cells/ml.

IMMUNOBEAD DETECTION AND SELECTION OF TUMOUR CELLS

Monoclonal antibodies and beads

MOC-31, an IgG1 class antibody that binds to an epithelial cluster 2 antigen (EGP-2) in carcinoma cells[23] (kindly provided by Dr L de Leij, University of Groningen, The Netherlands) was conjugated to magnetic sheep antimouse IgG M-450 Dynabeads (Dynal, Oslo, Norway) and used for magnetic detection and selection of tumour cells. As a negative control we used immunobeads with an isotype matched antibody that does not bind to epithelial cells (9.2.27; a generous gift from Dr R Reisfeld, Scripps Research Institute, La Jolla, California, USA).

Immunomagnetic separation

In both model experiments and analysis of clinical samples, mononuclear cells were resuspended to a final concentration of 10^7 cells/ml and transferred to 10 ml round bottom polystyrene tubes (Nunc, Naperville, Illinois, USA). All solutions were kept on ice during the whole procedure to avoid non-specific binding of the immunobeads. The antibody conjugated beads were added at a ratio of 2:1 to total number of cells, and the suspension was incubated for 30 minutes at 4°C on a rotating mixer. After incubation, the cells were diluted with PBS + 1% HSA and placed in an MPC magnet holder (Dynal) for approximately two minutes. The supernatant, containing unbound cells, was decanted and the remaining positive fraction, ~ 200 µl, was placed on ice. A 20 µl aliquot of the positive fraction was transferred to a Bürker counting chamber for microscopic detection and counting of cells with more than five immunobeads bound to their surface (bead rosettes). The positive fraction, and in some cases also the supernatant (negative fraction), was kept on ice for the preparation of interphase nuclei.

FISH IN INTERPHASE NUCLEI

Preparation of interphase nuclei

Frozen tumour tissue was pulverised in liquid nitrogen, transferred to a centrifuge tube, and immediately fixed in methanol/acetic acid (3/1), whereas cell suspensions were treated with hypotonic (0.05 M) KCl for 30 minutes at 37°C, then fixed. After centrifugation, the pellets were resuspended in a small volume of 60% acetic acid. Several preparations were made from each sample. A few drops of the suspension were applied on to Menzel Superfrost slides prewarmed to 45–50°C and left to dry at the same temperature. Slides were stored at −20°C before use. Each of the positive bone marrow specimens was diluted to make several slides; hence, each slide contained relatively few cells. This was done to allow analyses of many different genes.

FISH

Slides were thawed and immersed in 75% ethanol at 4°C for 1–2 hours before use, then air dried and denatured in 70% formamide in 2× saline sodium citrate (SSC), pH 8.0, for three minutes at 74°C, washed three times in ice cold 2× SSC, dehydrated, and air dried. Thereafter, slides were treated with proteinase K (0.1 µg/ml in 20 mM Tris/HCl, 2 mM $CaCl_2$, pH 7.0) for seven to 10 minutes at room temperature, washed again in 2× SSC, dehydrated, and air dried. Probes from Vysis (Downers Grove, Illinois, USA) were diluted in

a solution of 50% formamide, 10% dextran sulphate, and 2× SSC, denatured for five minutes at 75°C, and incubated for two minutes at 45°C. Other probes were prewarmed for five minutes at 37°C and then applied to slides at room temperature. Hybridisation was performed overnight at 37°C. After hybridisation, the slides were washed three times for 10 minutes in 50% formamide in 2× SSC at 45°C, and then three times for 10 minutes in 2× SSC at 60°C, and once in 2× SSC at room temperature. For detection of the biotin labelled int2 probe and the digoxygenin labelled erbB2 probe we used avidin conjugated CY3 (Amersham Life Science, Little Chalfont, UK) and/or fluorescein isothiocyanate (FITC) conjugated antidigoxygenin (Boehringer Mannheim, Mannheim, Germany), respectively. After dehydration, the slides were dried, counterstained with 4',6-diamino-2-phenylindole (DAPI), and mounted in "antifade" solution.

Evaluation of results

The slides were analysed using a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped with appropriate single bypass filters for excitation of DAPI and FITC (spectrum green); double bypass filters for excitation of DAPI/rhodamine (CY3 and spectrum orange) and DAPI/FITC (spectrum green); and single filters for excitation of DAPI or FITC. The slides were viewed with a ×63 or ×100 Plan Apochromat lens (Carl Zeiss) using DAPI excitation to localise the areas with interphases. Nuclear boundaries were determined by DAPI excitation at the same magnification, and nuclei that were either partially or totally overlapping or not intact were not analysed. In spiking experiments, the copy numbers of erbB2, int2, and centromere 17 were examined in a minimum of 150 nuclei from different randomly chosen areas of the preparation. For the positive selections from model experiments with the lowest proportions of tumour cells (10^{-5} and 10^{-6}), the whole preparation was examined to find >150 cells.

FISH probes

Spectrum Orange (LSI^{TM}HER-2/neu; Vysis) or digoxygenin (P5111-DG.5; Oncor, Gaithersburg, Maryland, USA) labelled probes were used to determine amplification of the erbB2 locus at chromosome 17q11.2–q12. Chromosome 17 copy numbers in the tumour were evaluated using a spectrum green labelled centromeric α-satellite probe (CEP 17; 17p11–q11) specific for this chromosome (Vysis). For detection of int2 amplification we used a biotin or digoxygenin labelled probe specific for genomic sequences including the FGF3 locus at 11q13.3 (Oncor).

Results

IMMUNOBEAD SELECTION AND FISH ANALYSIS IN MODEL EXPERIMENTS

Initially, we used a model system where a known quantity of cells with amplification was mixed with mononuclear cells in defined proportions. erbB2 is known to be amplified and overexpressed in the SKBR3 cell line, and previous experiments have shown that the pan-epithelial mucin antibody MOC-31[23] conjugated to immunobeads binds well to the SKBR3 cell line, with ~ 80% of the cells staining positively (HK Høifødt and Ø Fodstad, unpublished results, 1994).

Table 1 Model experiments determining the detection level of tumour cells in SKBR3/MNC (mononuclear cell) suspensions

Proportion of SKBR3 cells among MNCs	*Rosetted cells binding ⩾5 immunobeads*
$10^4/10^7$	810
$10^3/10^7$	590
$10^2/10^7$	48
$10^1/10^7$	5

In FISH experiments, almost all SKBR3 nuclei (95 of 100) showed amplification of erbB2, although not always at the same level (in most cases, the number of signals could not be counted, although some cells had seven to 10 signals). Increased copy numbers of int2 were also found in most cells but at lower levels (four to six signals). However, int2 and erbB2 were always co-amplified (results not shown).

SKBR3 cells were then mixed with 10^7 mononuclear cells from peripheral blood to give 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} tumour cells to mononuclear cells, and MOC-31 positive cells were extracted using immunomagnetic beads. The positively selected tumour cells were studied by light microscopy, and tumour cell numbers were measured by counting the numbers of immunobead rosetted cells in the suspensions, as described (table 1).

FISH analysis of erbB2 and int2 copy numbers was performed in parallel on the different unselected SKBR3/mononuclear cell suspensions and on positively selected fractions. In addition, erbB2 copy numbers were determined in the fraction remaining after immunobead selection from the 10^3:10^7 mixture. On each slide, we counted about 200 interphase nuclei with and without amplification in different randomly chosen areas.

In the unselected suspensions, cells with amplification were difficult to detect because large numbers of normal cells surrounded or covered the tumour cells (not shown). However, upon very thorough examination of the slides we could identify some cells with erbB2 or int2 amplification in suspensions where there were 10^4 and 10^3 SKBR3 cells to each 10^7 mononuclear cells, but none at all when only 10^2 and 10^1 SKBR3 cells were present for each 10^7 mononuclear cells (table 2).

By contrast, in the positively selected fractions, cells with erbB2 or int2 amplification were detected even when a maximum of 100 and 10 SKBR3 cells were present (fig 1A–C; table 2). Moreover, in general, the tumour cells were much easier to detect because there were far fewer normal cells present.

We also checked the negative fraction from the 10^3:10^7 SKBR3 to mononuclear cell suspension for the presence of cells with erbB2 amplification, and did detect some (not shown). This result was not unexpected because only ~ 80% of SKBR3 cells express the MOC-31 antigen (Ø Fodstad and

Table 2 Model experiments demonstrating the increased sensitivity of FISH analysis after immunomagnetic selection of target cells

Proportion of SKBR3 cells among MNCs	*Nuclei with detectable amplification*			
	erbB2		*int2*	
	Unselected	*Immunobead selected*	*Unselected*	*Immunobead selected*
$10^4/10^7$	16/228	43/221	7/208	32/194
$10^3/10^7$	4/214	31/193*	3/215	10/163
$10^2/10^7$	0/226	4/189*	0/200	3/156
$10^1/10^7$	0/226	2/200	0/200	2/202

Interphase nuclei with increased erbB2 and int2 copy numbers/number of counted nuclei before and after immunomagnetic selection of target cells.
*Mean of two experiments.
MNCs, mononuclear cells.

HK Høifødt, unpublished results, 1994). However, in these samples it was difficult to detect the cells with amplification because of the large numbers of normal cells.

FISH ANALYSIS OF erbB2 AND int2 AMPLIFICATION IN PRIMARY TUMOUR MATERIAL

To test the approach on patient samples, we first analysed tumour tissue from 15 patients with advanced breast cancer to identify samples with erbB2 and/or int2 amplification. In addition, we used a probe for centromere 17, but all tumours had a normal copy number of this probe (two signals, not shown), and therefore it was not included in further analyses.

As shown in table 3, eight of the 15 tumour samples had amplification of erbB2 and/or int2, and in five of these, both genes were amplified. Differentially labelled int2 and erbB2 probes were hybridised simultaneously to interphase nuclei from BC19 and BC37 to determine the frequency of co-amplification. In both samples, we always saw co-variation in copy numbers (not shown) because both

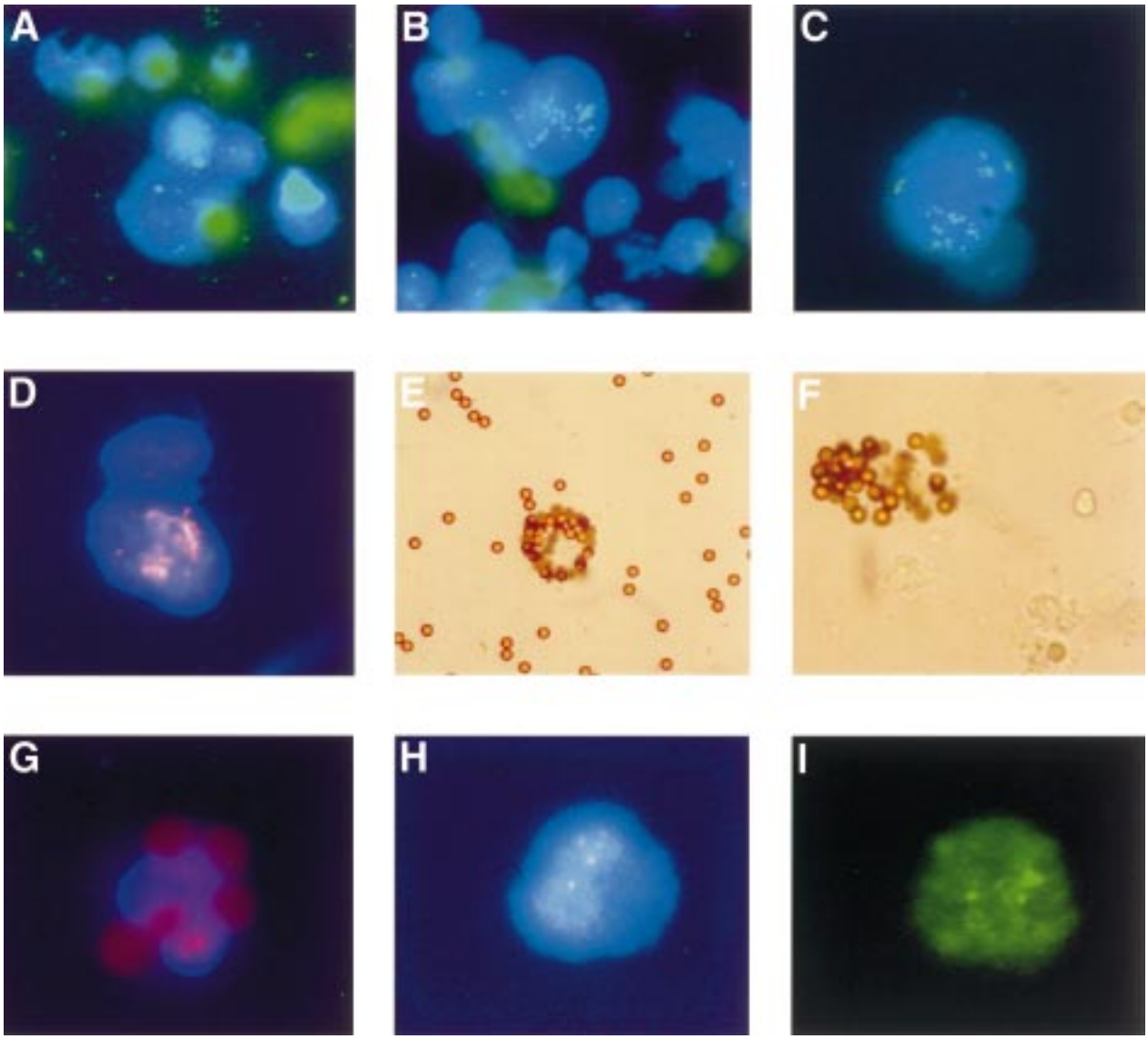

Figure 1 FISH analysis and immunomagnetic selection of tumour cells from model experiments and primary tumours/bone marrow samples. (A–C) Model experiments. Interphase nuclei prepared after immunomagnetic selection of SKBR3 cells mixed with 10^7 mononuclear cells at ratios of 10^{-5} (A and B) and 10^{-7} (C). Cells with increased copy numbers of int2 (A) and erbB2 (B and C) could be found in all the positively selected fractions. (D–F) Tumour and bone marrow samples. (D) Interphase nuclei from primary tumour BC19 showing high level amplification of erbB2. (E and F) Rosetted tumour cells from bone marrow of patients BC19 (E) and BC3 (F) after immunomagnetic selection (+ selection). (G–I) FISH analysis of tumour cells selected from bone marrow samples BC19+ (G and H) and BC3+ (I). Interphase nuclei from BC19+ show high level amplification of erbB2 (G) and five signals with int2 (H). Interphase nuclei from BC3+ show increased copy numbers (three signals) with erbB2. The small red or green coloured "bullets" are the immunomagnetic beads.

Table 3 Primary tumour samples with amplification of erbB2 and/or int2 in more than 5% of the analysed nuclei

Tumour	erbB2 amplification (percentage of cells)	int2 amplification (percentage of cells)	IMS bone marrow (number of rosetted cells)
BC3*	3–10 s (20–30)	3–4 s (10–20)	BM positive (4000)
BC5	3–4 s (20–30)	3–4 s (10–20)	BM negative†
BC8	3–10 s (10–20)	3–10 s (10–20)	BM negative
BC9*	Normal	10 s (20–30)	BM positive (26)
BC19*	High level (70)	6 s (70)	BM positive (540)
BC37	3–4 s (30–40)	3–4 s (30–40)	BM negative
BC50	High level (30–40)	Normal	NA
BC67	Normal	3–10 (19)	BM negative

Results of FISH analyses of the primary tumour samples. Patients with amplification of erbB2 and/or int2 in > 5% of the analysed nuclei were analysed further for the presence of tumour cells in the bone marrow by immunobead selection (IMS).
*Samples with amplification of erbB2 and/or int gene and positive bone marrow as determined by IMS.
†IMS detected no rosetted cells, but one cell with three copies of int2 was detected by FISH analysis of the bead containing fraction.
s, spots; BM, bone marrow; NA, not analysed—bone marrow not available.

probes gave either amplified or normal signals.

In general, erbB2 showed higher copy numbers than INT2, and copy numbers were variable, ranging from high level amplifications, where the number of spots could not be counted, to small increases (three to four spots). In addition, the proportion of cells with amplification in each sample was variable, possibly indicating tumour cell heterogeneity. A similar variation in amplification patterns among cells in the same tumour has also been observed for sarcomas.[24]

IMMUNOBEAD ENRICHMENT AND FISH ANALYSIS OF TUMOUR CELLS IN BONE MARROW SAMPLES

Selection and quantitation of tumour cells

The immunobead selection method was used to examine bone marrow aspirates for the presence of tumour cells. Bone marrow samples from the eight patients with erbB2 and/or int2 amplification in their primary tumour, as well as two samples with normal copy numbers of the genes (BC7 and BC16) were checked. In three of the bone marrow samples, tumour cells could be detected and quantified (table 3). In the sample from BC3, the estimated number of tumour cells using the MOC-31 immunobeads was ~ 4000 rosetted cells/10^7 mononuclear cells, for BC19 the number of tumour cells was 540/10^7 mononuclear cells, and for BC9 it was only 26/10^7 mononuclear cells. Examples of rosetted cells are shown in fig 1E and F. In the sample from BC5, cells were aggregated and we were unable to decide whether tumour cells were present. No cancer cells were found in the bone marrow samples from the other three patients with detectable amplification in their primary tumours—BC8, BC37, and BC67—or in BC7 and BC16. Frozen blood or bone marrow from BC50 was not available.

FISH analysis on selected tumour cells

We performed FISH analysis of the positively selected fractions (+) from the three bone marrow samples with detectable micrometastatic cells (BC3+, BC9+, and BC19+), and of the negative fraction from BC19. In addition, we analysed the selected fraction from BC5.

Cells with amplified erbB2 or int2 were detected in the positive selections BC3+ and BC19+, despite the presence of numerous cells with normal signals. In BC19+, the tumour cells had high levels of erbB2 (fig 1G) and somewhat lower levels of int2 amplification (fig 1H), in agreement with the findings in the primary tumour tissue. On one slide we could find three cells with high level amplification of erbB2 among 13 analysed; another slide had 11 positive cells, but also a large number of normal cells. Slides hybridised with int2 always showed two or three cells with three copies among the normal cells.

As expected, in BC3+ we detected tumour cells with three copies of erbB2 (six cells with three signals among the 98 cells analysed) (fig 1I) or int2 (three cells with three signals among the 48 analysed). The bead containing fraction from BC5 bone marrow was also analysed. Although only a few cells were present, we were able to detect one cell with three copies of int2 among the normal cells, but no cells with erbB2 amplification (not shown). It is likely that tumour cells were present in this sample although, for the reasons described above, we were unable to determine their numbers by the immunobead method. Owing to the poor quality of the interphase nuclei from BC9 we could not count the numbers of erbB2 or int2 copies in this sample.

Discussion

In several types of cancer, an association has been noted between early relapse and the presence of tumour cells in the bone marrow at the time of surgery.[3 5 6] In view of this, detection of circulating malignant cells would clearly be of value as a predictor of prognosis. However, it is not known whether all such tumour cells have a phenotype that enables them to give rise to distant metastases, or whether they are heterogeneous with regard to their metastatic capacity. Alternatively, they could be unselected cells that are released from the primary tumour upon surgery, but are cleared away shortly afterwards. Therefore, it seems important to study micrometastatic cells for characteristics associated with poor prognosis. In breast cancer, overexpression of erbB2 and int2 appears to be indicative of aggressive disease. Gene overexpression is often a result of gene amplification, and we have developed a technique for the analysis of erbB2 and int2 amplification in circulating malignant cells by combining immunomagnetic selection and FISH analysis.

The immunobead rosetting method, used here for detection and enrichment of micrometastatic cells in model experiments and in bone marrow samples from patients with breast cancer, can be applied to any cancer type with cells expressing antigens not present on normal mononuclear cells or bone marrow cells. Furthermore, the method is superior to immunohistochemistry with respect to sensitivity, simplicity, and speed,[8 9] and the selected cells can be propagated in culture and used in further experiments[25] or used for FISH analysis, as shown here.

For several reasons, FISH analysis of genetic aberrations is better than methods such as

Southern blot analysis[21] and the polymerase chain reaction (PCR). Specificity and sensitivity are high, and many kinds of genetic alterations can be analysed. Furthermore, several alterations can be analysed simultaneously using differentially labelled fluorescent probes. In contrast to PCR, FISH allows analysis of individual cells, and amplification levels can be quantified. Recently, Zojer *et al* showed that FISH analysis also improved the detection of malignant cells in effusions from breast cancer patients when compared with routine cytology.[26]

Our model experiments show that FISH performed on target cells enriched and detected by the immunobead method can be particularly useful because it allows quantification as well as molecular characterisation of the circulating tumour cells. FISH analysis of positively selected fractions could detect cells with amplification of erbB2 and int2 with as few as 10 tumour cells (SKBR3) present among 10^7 mononuclear cells. Compared with the lowest ratio (10^3:10^7 cells, respectively) at which cells with amplification could be found in non-enriched samples (table 2), the sensitivity of the combined approach is very high.

Mueller *et al* recently published a similar method for the detection and molecular characterisation of epithelial cells in bone marrow aspirates from prostate carcinoma patients.[27] Their approach uses cytokeratin antibodies to detect micrometastases in bone marrow cytospins, and FISH for characterisation of chromosome 1, 7, and 8 aneusomy, which is frequently found in such cancers. Detection of cytokeratin positive cells requires the screening of 12 slides for each patient. In comparison, when using immunomagnetic selection, positive cells can be detected by screening of one slide only. In addition, rosetted cells are detected easily among the non-rosetted ones, and this part of the procedure can be performed within two hours.

Another advantage of our approach lies in the specificity of the EGP-2 antigen for epithelial cells. Although in spiking experiments the antibody does not bind to 100% of SKBR3 cells, screening of antibody reactivity to normal and malignant cells and tumours[23 28 29] shows a broad and consistent expression pattern in carcinomas and not in normal haematopoietic cells. The other method uses cytokeratin 8 and 18 to identify tumour cells, but because the sensitivity of direct immunocytochemistry is limited,[30] and also because these antigens can be expressed on bone marrow cells,[31] immunohistochemistry alone may not be optimal in confirming the presence of micrometastases in bone marrow.

FISH can be used for confirmation and characterisation of micrometastases. However, because FISH is performed on interphase nuclei, antibody labelling is lost, and tumour cells with normal gene or chromosome copy numbers cannot be identified. Thus, the true frequency of tumour cells with amplification in the bone marrow cannot be determined. Likewise, heterogeneity among the tumour cells found in bone marrow may not be detected if some of the former have normal copy numbers of the markers used.

Our method was tested on a limited number of clinical bone marrow samples from patients with breast cancer (table 3). By immunomagnetic selection, we detected malignant cells in bone marrow in three of the eight patients with erbB2 and/or int2 amplification in their primary tumour (BC3, BC9, and BC19), whereas the remaining five were negative. Most likely, very few tumour cells were present in the bone marrow of these patients. Bone marrow samples from two other patients were checked as well, but were found to be negative. FISH analysis detected cells with erbB2 and int2 amplifications in the positive selection from BC3 and BC19 bone marrow samples, whereas BC9 could not be properly examined because of the poor quality of the nuclei. However, we could detect one cell with an increased copy number of int2 in the positive fraction of BC5.

The rationale for establishing this assay was the possibility that the genetic makeup or other characteristics of the micrometastatic cells would be more important than their numbers in determining the clinical outcome. We did not have enough samples to draw any conclusions about this but it is interesting that patient BC19, who had a primary tumour with high level amplification of erbB2 in nearly 70% of cells (which was also found in the bone marrow), had a particularly aggressive form of the disease. Multiple bone metastases were detected only five months after the primary, inoperable tumour was diagnosed, and the patient died shortly afterwards. The number of tumour cells in the bone marrow of this patient was, however, not more than $540/10^7$ mononuclear cells, as detected by immunomagnetic rosetting. In contrast, patient BC3, in whom we detected about 10 times the number of tumour cells in the bone marrow, had no relapse or metastases five years after surgery. This patient had a small increase of erbB2 (and int2) copy numbers in a smaller fraction of the primary cells, and only three copies of erbB2 or int2 in the circulating tumour cells. Thus, further characterisation of micrometastatic cells may be relevant. Until now, this aspect has received limited attention,[6] mainly because of technical limitations inherent in the methods used, and also because of the low number of cells available for analysis in unselected cell populations in bone marrow aspirates.

Because the immunomagnetically selected fractions contain many unbound immunomagnetic beads that tend to cluster on and round the nuclei, it was impossible to analyse all tumour cells present. Detachment of beads from the tumour cells before preparation of interphase nuclei might improve the accessibility of cells for in situ hybridisation.

The combination of immunomagnetic selection and FISH in bone marrow samples from patients with cancer might open possibilities for more accurate prediction of prognosis based on the characteristics of micrometastatic cells. Further studies on larger clinical series should be performed to determine whether the presence of micrometastatic cells with int2 or erbB2

amplification, compared with cells without such alterations, are indicative of increased risk of relapse and/or poorer survival of the patients.

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