Eur J Cancer, Vol. 27, No. 3, pp. 236–239, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Micrometastases in Breast Cancer: Long-term Follow-up of the First Patient Cohort

D.P. Dearnaley, M.G. Ormerod and J.P. Sloane

"Micrometastases" can be identified in the bone marrow of patients with apparently localised breast cancer using an immunocytochemical stain for epithelial membrane antigen (EMA). Of 39 women who had marrow samples examined at the time of initial presentation (37), or with locally recurrent disease (2), 13 (33%) had samples which contained small numbers of EMA positive cells. 10 out of 23 (44%) lymph-node positive patients were marrow positive, compared to 1 out of 14 (7%) lymph node negative cases ($P = 0.03$). Long-term follow-up (median 9.5 years) has shown that 11 out of 13 (85%) patients with micrometastases have developed metastatic disease compared to 8 out of 26 (31%) with negative bone marrow aspirates ($P < 0.05$). The small number of EMA positive cells detected in bone marrow samples probably reflects the high metastatic potential of primary or recurrent cancers rather than established microscopic deposits; it is not yet clear whether the finding of such micrometastases will act as an independent variable compared to established prognostic factors.
Eur J Cancer, Vol. 27, No. 3, pp. 236–239, 1991.

INTRODUCTION

WE FIRST reported an immunocytochemical method capable of increasing the detection of carcinoma cells in bone marrow samples taken from women with breast cancer in 1981 [1]. Small numbers of morphologically malignant cells were identifiable using an antiserum to epithelial membrane antigen (EMA) which could not be demonstrated using conventional techniques. Our initial studies on women presenting with localised primary breast cancer [1, 2] suggested that about 30% of bone marrow samples contained positively staining cells and that their presence predicted the development of distant and in particular bone metastases. Further studies from the Royal Marsden and St George's Hospital Group [3–6] have confirmed these findings in a larger cohort of patients and initial follow-up indicates that EMA marrow positivity closely relates to other prognostic features including tumour size, lymphovascular invasion and lymph node status, and that the presence of cells staining for EMA predicts for a shorter relapse-free interval and reduced overall survival. However, the number of positive cells detected, even when samples are aspirated from multiple sites, is very small and it is uncertain whether all patients will go on to develop established metastatic disease. This report details long-term follow-up of the first cohort of patients investigated by our group.

PATIENTS AND METHODS

Bone marrow samples were taken from 39 women with breast cancer who presented to the Royal Marsden Hospital between March 1980 and May 1981. Clinical staging is shown in Table 1. 37 patients were studied immediately prior to surgery for localised primary breast cancer and 2 prior to excision of locally recurrent disease. The median age of patients was 60.5 years (range 38–84 years), 13 women were pre- or perimenopausal and the remaining 26 postmenopausal. Each patient was screened for metastatic disease as previously described [7] by clinical examination, measurement of plasma alkaline phosphatase and γ-glutamyl transferase, bone scan and skeletal survey. Additionally, liver ultrasound was performed if hepatomegaly or abnormal liver function was noted. Patients with primary breast cancer were treated according to the protocols in use at the time. Medial and lateral cancers were treated by local excision followed by post-operative radiotherapy to the breast, axilla, supraclavicular fossa and internal mammary chain. Radical mastectomy was employed for central tumours or when conservative surgery would result in poor cosmesis. Postmenopausal patients with involved axillary lymph nodes where entered into an adjuvant trial comparing aminoglutethimide with placebo. Premenopausal and axillary node negative postmenopausal patients did not receive adjuvant therapy.

Bone marrow samples

18 patients had single bone marrow aspirates taken from the posterior iliac crest (mean volume 1.0 ml) and 21 women had mutliple samples taken from six sites (sternum × 2, anterior

Table 1. Relationship of initial clinical stage, marrow micrometastases and outcome

	Clinical stage					
	Primary disease					
	T_1/T_2	T_3/T_4	Node negative	Node positive	Local recurrence	Total
Total no.	20 (10)	17 (7)	14 (7)	23 (10)	2 (1)	39 (18)
Development of metastases	7 (3)	11 (4)	1 (0)	17 (7)	1 (1)	19 (7)
Marrow status*						
EMA−	3/15	5/11	0/13	8/13	—	8/26
EMA+	4/5	6/6	1/1	9/10	1/2	11/13

No. in parentheses represents patients who had single marrow aspirates.
*Metastases/no.

Correspondence to D.P. Dearnaley.
The authors are at the Academic Radiotherapy Unit, Royal Marsden Hospital, Sutton, Surrey SM2 5PT, U.K.
Revised 13 Dec. 1990; accepted 19 Dec. 1990.

and posterior iliac crests bilaterally—mean volume 7.1 ml). Additionally, a bone marrow trephine biopsy was taken from the left posterior iliac crest together with 15 ml of peripheral blood from an anticubital vein.

Bone marrow smears were prepared for immunocytochemical staining as previously described [1, 2]. Briefly, erythrocytes were removed on Ficoll–Hypaque prior to washing, smearing and wet fixing the cell preparations in 95% ethanol. Smears were stained using an immuno-alkaline phosphatase method. The primary rabbit antiserum was raised against human milk fat globule membrane and absorbed to render it specific for EMA [8, 9]. The second antibody was affinity purified sheep anti-rabbit immunoglobulin, conjugated to alkaline phosphatase. Endogenous alkaline phosphatase activity in osteoblasts and leucocytes was blocked by pre-incubation of the slides with 20% acetic acid and 2.28% periodic acid in addition to adding 1 mmol of levamisole to the chromagen substrates (brentamine fast red TR and Napthol AS:BI). Specificity of staining was demonstrated by absorbing antiserum with a purified preparation of EMA [10].

Bone marrow smears were screened by a single observer (DPD) and scored positive when cells stained strongly for EMA and had the morphological characteristics of malignant cells. In 32 aspirates from patients without epithelial malignancy, occasional staining was seen on plasma cells, early myeloid cells and degenerate cells [2, 11, 12]. Using monoclonal antibodies raised against milk fat globule membrane [13] we have shown that this is due to the weak expression of a determinant of the epithelial membrane antigen by these cells and not due to an impurity in antibody [14]. No "false positive" results were given when these 32 control smears were screened "blindly" amongst the smears from patients with breast cancer.

Follow-up

Patients were followed initially at 3 monthly intervals during year 1, at 6 monthly intervals during years 2–5 and annually subsequently. Chest X-ray, alkaline phosphatase and γ glutamyl transferase were performed at each attendance. Patients were fully re-evaluated with bone scan, skeletal survey and liver ultrasound if metastases developed. Median follow-up of survivors is 9.5 years (range 8–10 years).

RESULTS

Relationship of micrometastases to other presenting features

EMA positive cells were found in bone marrow aspirates from 13 of the 39 (33%) patients. The median number of positively staining cells detected was 10 (range 1–80). Of patients who had single aspirates taken, 4 out of 18 (22%) were positive compared to 9 out of 21 (43%) cases who had multiple bone marrow samples. These groups contained patients with tumours of comparable stage; of the 17 women in the single aspirate series 7 patients had T3/4 tumours, 10 cases axillary node involvement and 1 patient had locally recurrent disease; of the 20 women in multiple aspirate series 10 patients had T3/4 lesions, 13 cases axillary node involvement and 1 patient had locally recurrent disease (Table 1).

Overall 10 out of 23 (44%) women with primary breast cancer who had positive axillary nodes had positive marrow samples compared to 1 out of 14 (7%) for node negative patients ($P = 0.03$, Fisher's exact test). Patients with small primary tumours (histological diameter ≤ 2 cm) were less likely to have micrometastases than women with larger tumours with positive marrow samples found in 3 out of 18 (17%) and 8 out of 19 (42%) respectively ($P = 0.15$). There was no clear relationship between marrow positivity and oestrogen receptor (ER) status in the 22 patients who had receptor assays performed. 3 out of 14 women with ER positive tumours (>10 f mol/mg cytosol protein) had positive marrows compared with 3 out of 8 with ER negative tumours. There appeared to be little difference in the immunocytochemical staining characteristics of the primary tumour for EMA between those patients with positive or negative bone marrow results. The majority of carcinoma cells (>80%) stained positively for EMA in 20/39 (51%) of the breast cancers studied, but the only two specimens which had <20% of breast carcinoma cells staining had negative bone marrow aspirates.

Significance of EMA positive cells on follow-up and relationship to other prognostic factors

Of the 39 patients, 19 have developed distant metastases and 18 have died from disseminated breast cancer. A single woman remains alive with active disease. An additional 4 patients have died from intercurrent illness (cardiovascular disease (2), carcinoma of the rectum, and carcinoma of pancreas) 33–96 months after initial investigation. 11 of the 13 (85%) patients with EMA positive cells have developed metastases but the remaining 2 women with positive samples have remained free of disease on follow-up for over 9 years. One of these patients presented with a T3a N1a breast carcinoma and was treated with radical mastectomy and axillary lymph node dissection (histologically 3 out of 27 lymph nodes contained metastatic cancer) followed by postoperative irradiation; the other patient had a bone marrow aspirate taken at the time of excision of an isolated local recurrence. Neither case received adjuvant systemic treatment. This compares to 8 out of 26 (31%) recurrences in the women with negative marrow aspirates. Acutarial disease free and survival curves (Fig. 1) demonstrate a significantly worse outcome for patients with EMA positive bone marrows ($P < 0.05$). The median time to recurrence and death from breast cancer was shorter for the marrow positive group at 10 months vs. 24 months and 23 months vs. 47 months respectively. In the EMA positive group the first site of metastases included bone in 10 out of 11 (91%) of cases but involved both osseous and extra-osseous sites in 7 out of 11 (64%) of cases. All but 2 of these women eventually developed metastases at extra-osseous sites. 4 out of 8 (50%) of the EMA negative group relapsed at non-osseous sites alone initially and 3 remained without clinical or radiological evidence of bone metastases on follow-up.

As expected patients with axillary lymph node involvement or advanced primary breast cancers had an increased risk of developing metastases (Table 1). The best predictor of disease free survival was axillary lymph node status and only 7% (1 out of 14) of node negative patients developed recurrence compared to 74% (17 out of 23) for women with involved nodes. Patients with EMA positive cells in the bone marrow had the least favourable outcome and 91% (10 out of 11) have developed recurrent disease compared to 31% (8 out of 26) with negative marrows. Combining these factors identified 3 groups: (i) lymph-node negative, EMA negative; (ii) lymph-node positive, EMA negative; (iii) lymph-node negative or positive, EMA positive. Recurrent disease has developed in 0% (0 out of 13), 62% (8 out of 13), and 91% (10 out of 11) of each group respectively. Initial T stage or pathological size of the primary tumours did not contribute further to this stratification.

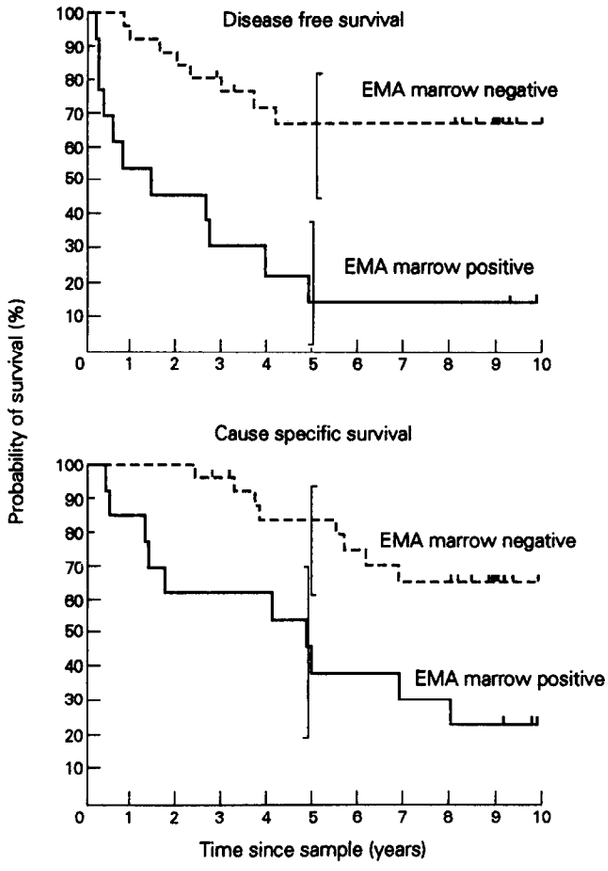

Fig. 1. Comparison of patients with positive or negative bone marrow aspirates taken at the time of surgery for primary breast cancer or locally recurrent disease.

DISCUSSION

Long term follow-up of this cohort of patients has confirmed our previous observations that the presence of small numbers of EMA positive cells in bone marrow is associated with a high risk of development of metastatic disease and poor long-term prognosis. The median mean time from bone marrow aspiration to development of metastases was 10 months (range 3–58). As previously reported there is a close association between well documented prognostic factors such a lymph node status, size of primary tumour and presence of peritumoural lymphovascular invasion with EMA marrow positivity and it is not yet clear whether the finding of marrow "micrometastases" will act as an independent prognostic factor [2–6]. In this small series EMA marrow status appeared to stratify lymph node positive patients into a very high risk group for recurrence (9 of 10 marrow positive, lymph node positive cases developed metastases) and a moderate risk group (8 of 13 marrow negative lymph node positive cases developed metastases). Although none of the marrow negative lymph node negative cases recurred, the only patient with a positive marrow but negative lymph nodes developed metastases. Further follow-up of larger cohorts of patients will clarify these observations.

Of major interest is the explanation of apparently false positive or false negative results. In this series, 2 out of 13 patients with EMA positive cells remain free of disease over 9 years after initial testing. There are a number of possible explanations. The EMA positive cells detected could represent circulating tumour cells (however, it is unlikely that such cells have prognostic importance [15]); a "false positive" staining reaction of normal bone marrow cells; or breast cancer cells which have settled in the marrow but which do not necessarily have the clonogenic capacity to form established metastases. Finally, although follow-up is mature, further recurrences may yet appear. The explanation of circulating tumour cells is unlikely as in our series of patients peripheral blood samples taken at the same time as marrow aspirations were always negative. Weak "false positive" staining reactions may occur on plasma cells and early myeloid and lymphoid cells [2, 10, 11] using antiserum to EMA. The reaction occurs with both polyclonal and monoclonal antibodies and is abolished by absorption of antibodies with a preparation of EMA [10] indicating the expression of a shared epitope. However, in contradistinction to the finding of Thor and colleagues [16] we found no difficulty in distinguishing these patterns of staining in normal marrow samples. This is likely to be due to the difference in antiserum used and emphasises the importance of utilising normal bone marrow control samples in this type of study. We favour the third explanation. It is likely that the finding of initial bone marrow positivity identifies a subset of patients whose primary tumours have the potential to seed carcinoma cells widely throughout the bone marrow. However, only a small proportion (if any) of these cells will be clonogenic with the capacity to establish metastases.

This hypothesis is supported by additional studies [17] which have re-examined patients with further bone marrow aspirates at a median time of 18 months after initial testing. Only 2 out of 21 (10%) of patients with micrometastases at presentation remained with positive marrow examinations. Nevertheless our findings on long term follow-up suggest that the remaining 90% of patients with negative marrows will remain at high risk of developing disseminated breast cancer. This suggests firstly that the primary tumour was the source of the carcinoma cells in the marrow at initial presentation and that the majority of these cells are non-viable, and secondly that our bone marrow assay is insufficiently sensitive to reliably detect established deposits at an early stage of their development. Not only may these be present in small numbers but once metastases become established they may be more difficult to aspirate becoming more firmly attached to the bone matrix; only a "direct hit" with the aspiration needle producing positive samples. In this regard it is interesting that we have not found immunocytochemical staining of bone marrow trephines of value in detecting micrometastases [2, 14] and it is likely that this is due to the smaller volume of marrow sampled with this technique. If, as suggested, initial bone marrow positivity is an expression of high metastatic potential, rather than a test detecting minute but established microscopic deposits, it is not surprising that a few patients should remain free from recurrence even after long term follow-up.

The second issue is of "false negative" results. So far, 8 out of 26 of our EMA negative cases have developed metastatic disease. Although 3 of these patients developed locoregional recurrence or a second primary breast cancer before the detection of metastases the remaining 5 women had no locoregional recurrence and metastases must have been present at the time of initial marrow aspiration. The reasons for negative bone marrow results include the limitations on sampling imposed by bone marrow aspiration, loss of EMA positive cells in processing samples, and failure to recognise carcinoma cells because of hetrogeneity of EMA expression. We have previously shown that using multiple instead of single sites of aspiration doubles the number of positive samples obtained [2]. It is not practical

to further increase sampling significantly and this will remain a potential limitation on the techniques sensitivity. It is inevitable that some of the EMA positive cells are lost in the processing technique and also that small numbers of positive cells may be overlooked in the screening process. We have explored methods designed to concentrate the positive cells but this has led to an increased loss of cells in processing and therefore reduced overall sensitivity [18]. Automatic scanning of slides has also been studied with promising results but it is not anticipated that this will produce an increase in sensitivity, although significantly decreasing the considerable labour involved in cytological screening [19]. Since this study started further antibodies capable of identifying epithelial cells have become available. Other groups have used a variety of polyclonal and monoclonal antisera [16, 20–26], and in particular the addition of antibodies raised against cytokeratins increases the number of breast cancer cells detected when used in conjunction with antiserum to EMA [4, 21, 24]. Between 17 and 37% of patients with primary breast cancer have been shown to have micrometastases [4, 21–25] and estimates of the sensitivity of the techniques used range from an ability to detect one positive cell in 10^4 to 10^6 nucleated bone marrow cells [14, 17, 22, 23, 25, 26]. For optimal sensitivity it is likely that the use of panels of antibodies will prove ideal and these can be selected on an individual basis from the immunocytochemical staining patterns on the primary tumour or lymph node metastases.

Long term follow-up from other series with multivariate analysis comparing the significance of the finding of micrometastases with other prognostic factors will define the role of this technique in routine practice. The 91% recurrence rate in patients with primary breast cancer and positive bone marrows strongly suggests that this group is at particularly high risk for the development of metastases and it will be of interest to document the impact of adjuvant therapy on relapse rates and survival in this group in the larger patient cohorts currently under study.

1. Dearnaley DP, Sloane JP, Ormerod MG, *et al.* Increased detection of mammary carcinoma cells in marrow smears using antisera to epithelial membrane antigen. *Br J Cancer* 1981, **44**, 85–90.
2. Dearnaley DP, Ormerod MG, Sloane JP, *et al.* Detection of isolated mammary carcinoma cells in marrow of patients with primary breast cancer. *J R Soc Med* 1983, **76**, 359–364.
3. Redding WH, Coombes RC, Monaghan P, *et al.* Detection of micrometastases in patients with primary breast cancer. *Lancet* 1983, **ii**, 1271–1274.
4. Mansi JL, Berger U, Easton D, *et al.* Micrometastases in bone marrow in patients with primary breast cancer: evaluation as an early predictor of bone metastases. *Br Med J* 1987, **295**, 1093–1096.
5. Mansi JL, Powles TJ, Coombes RC. The detection and evaluation of bone marrow micrometastases in primary breast cancer. *Dev Oncol* 1987, **51**, 299–301.
6. Berger U, Bettelheim R, Mansi JL, Easton D, Coombes RC, Neville AM. The relationship between micrometastases in the bone marrow, histopathologic features of the primary tumor in breast cancer and prognosis. *Am J Clin Pathol* 1988, **90**, 1–6.
7. Coombes RC, Powles TJ, Abbott M, *et al.* Physical tests for distant metastases in patients with breast cancer. *J R Soc Med* 1980, **73**, 617–623.
8. Heyderman E, Steele K, Ormerod MG. A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissue. *J Clin Pathol* 1979, **32**, 35–39.
9. Sloane JP, Ormerod MG. Distribution of epithelial membrane antigen in normal and neoplastic tissues and its value in diagnostic tumor pathology. *Cancer* 1981, **47**, 1786–1795.
10. Ormerod MG, Steele K, Westwood JH, Mazzini MN. Epithelial membrane antigen: Partial purification, assay and properties. *Br J Cancer* 1983, **48**, 533–541.
11. Sloane JP, Dearnaley DP, Ormerod MG. Epithelial membrane antigen and lymphoid cells. *Lancet* 1985, **i**, 109–110.
12. Delsol G, Gatter KC, Stein H, *et al.* Human lymphoid cells express epithelial membrane antigen: implication for diagnosis of human neoplasms. *Lancet* 1984, **ii**, 1124–1128.
13. Foster CS, Dinsdale EA, Edwards PAW, Neville AM. Monoclonal antibodies to the human mammary gland II. Distribution of determinants in breast carcinomas. *Virch Arch Pathol Anat* 1982, **394**, 295–305.
14. Dearnaley DP. The detection of metastatic disease using immunochemical methods. MD thesis, University of Cambridge, Cambridge, 1983.
15. Salsbury AJ. Significance of circulating cancer cells. In: Stoll BA, ed. *Secondary Spread in Breast Cancer, Vol. 3. New Aspects of Breast Cancer*. Oxford, Heinemann, 1977.
16. Thor A, Viglione MJ, Ohuchi N, *et al.* Comparison of monoclonal antibodies for the detection of occult breast carcinoma metastases in bone marrow. *Breast Cancer Res Treat* 1988, **11**, 133–145.
17. Mansi JL, Berger U, McDonnell T, *et al.* The fate of bone marrow micrometastases in patients with primary breast cancer. *J Clin Oncol* 1989, **7**, 445–449.
18. Buckman R, Redding WH, Dearnaley DP, Smith S, Coombes RC. Evaluation of a rosetting method in detection of breast cancer cells. *Br J Cancer* 1984, **49**, 103–106.
19. Mansi JL, Mesker WE, McDonnell T, Van Driel-Kulker AM, Ploem JS, Coombes RC. Automated screening for micrometastases in bone marrow smears. *J Immunol Meth* 1988, **112**, 105–111.
20. Gugliotta P, Botta G, Bussolati G. Immunocytochemical detection of tumour markers in bone metastases from carcinoma of the breast. *Histochem J* 1981, **13**, 953–959.
21. Schlimok G, Funke I, Holzmann B, *et al.* Micrometastatic cancer cells in bone marrow: *in vitro* detection with anti-cytokeratin and in vivo labeling with anti-17-1A monoclonal antibodies. *Proc Natl Acad Sci USA* 1987, **84**, 8672–8676.
22. Cote RJ, Rosen PP, Hakes TB, *et al.* Monoclonal antibodies detect occult breast carcinoma metastases in the bone marrow of patients with early stage disease. *Am J Surg Pathol* 1988, **12**, 333–340.
23. Porro G, Ménard S, Tagliabue E, *et al.* Monoclonal antibody detection of carcinoma cells in bone marrow biopsy specimens from breast cancer patients. *Cancer* 1988, **61**, 2407–2411.
24. Untch M, Harbeck N, Eiermann W. Micrometastases in bone marrow in patients with breast cancer. *Br Med J* 1988, **296**, 290.
25. Ellis G, Ferguson M, Yamanaka E, Livingston RB, Gown AM. Monoclonal antibodies for detection of occult carcinoma cells in bone marrow of breast cancer patients. *Cancer* 1989, **63**, 2509–2514.
26. Taha M, Ordonez NG, Kulkarni S, *et al.* A monoclonal antibody cocktail for detection of micrometastatic tumor cells in the bone marrow of breast cancer patients. *Bone Marrow Transplant* 1989, **4**, 297–303.

Acknowledgements—This study was carried out in collaboration with the clinicians of the Combined Breast Unit at the Royal Marsden Hospital, Sutton. D.P.D. was supported by the Ludwig Institute of Cancer Research (London) and M.G.O. by project grants from the Medical Research Council and Cancer Research Campaign. We thank Prof. R.C. Coombes and Prof. A.M. Neville for their invaluable support and advice.