Breast Cancer Research and Treatment **15**: 21–26, 1990.

Report

Immunohistochemical staining of bone marrow biopsies for detection of occult metastasis in breast cancer

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Key words: breast carcinoma, bone marrow biopsy, cytokeratin, immunohistochemistry, occult metastasis

Summary

Immunohistochemical (IHC) techniques should allow for a greater detection of bone marrow micrometastasis in patients with breast carcinoma. We studied a series of bone marrow (BM) biopsies negative by conventional histologic techniques from 93 patients with breast carcinoma. Prior to this study, twelve BM biopsies, positive by conventional histology, were stained with a panel of monoclonal antibodies (MoAb), directed either against cytokeratin (KL1, AE1-AE3, CAM5–2) or epithelial membrane antigen (EMA, HMFG2). KL1 appeared to be the most sensitive of the markers used in the detection of metastases and is available commercially. It therefore was the only MoAb used with the series of 93 BM biopsies negative by conventional examination. Within this series, among 45 patients clinically suspected of having bone marrow metastasis but with BM biopsies negative by conventional staining, one case showing myelofibrosis stained positive with KL1 demonstrating isolated tumor cells. For the 48 patients without suspicion of bone marrow metastasis at initial diagnosis for breast carcinoma, KL1 revealed no marrow metastasis.

Single bone marrow biopsy techniques whether stained by conventional or IHC methods do not appear to be useful tests to detect occult bone marrow metastasis, especially at initial diagnosis of clinically M_0 breast carcinoma patients.

Bone and bone marrow are known to be the most frequent sites of metastatic predilection in breast carcinoma [1]. That this is a frequent occurrence in patients with operable breast carcinoma with positive axillary metastasis is evidenced by the poor ten year disease-free survival of patients with four or more positive nodes, the reason for which adjuvant chemotherapy is usually given [2]. A current therapeutic dilemma is the group of node-negative patients who overall suffer a 25% relapse rate in ten years, and for which no definite risk determination (and hence potential benefit of adjuvant therapy) exists [3]. If metastases are to become apparent in the future, then theoretically, a sensitive means of determination should be able to detect these micrometastases at time of initial diagnosis and explain the eventual relapse [4]. Bone marrow biopsy therefore should theoretically be an effective means of detecting these metastases [5, 6] although conventional histologic methods have not been useful in this quest [7–9].

Currently, with the use of monoclonal antibodies and immunohistologic (IHC) techniques directed against cytokeratin and epithelial membrane anti-

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gen (EMA) of carcinoma cells, the detection of foreign cells in aberrant locations such as bone marrow (BM) has been greatly facilitated.

IHC-detected carcinoma cells seen on BM aspirations have been shown to have a detrimental prognostic value [10]. By conventional techniques, metastases are more frequently discovered by BM biopsy than aspiration [6, 11–13], and therefore it would be important to determine if this detection could be further improved by the use of IHC techniques. Also, a single BM biopsy would be much simpler to accomplish than the series of bone marrow aspirations necessary for that technique. We undertook such a study, first to identify the most sensitive monoclonal antibodies (MoAb) and then to use these for detection of micrometastases in BM in both patients at initial diagnosis and patients with progressive disease.

Methods and material

Preliminary study on BM biopsies positive by conventional examination

At first, we compared the quality of the immunostaining of different antibodies directed against epithelial antigen. For this, we used 12 BM biopsies, positive by conventional techniques, performed on patients during disease progression. 5 biopsies were obtained between 1971–72 and 7 between 1986–1987.

Immunohistologic method

Biopsies were fixed in Bouin's solution, decalcified in Decal 2 H (Chemicals Corp.), and embedded in paraffin. Standard staining was performed on all specimens with hematoxylin-eosin-safran, Gordon Sweet and Giemsa methods.

For IHC, the peroxidase anti-peroxidase (PAP) technique was used [14]. After deparaffinization and rehydration, the sections were progressively incubated at room temperature with methanol containing 3% hydrogen peroxide for 15 min. to inhibit endogenous peroxidase; normal rabbit serum 1/20 (Dako), for 20 min.; monoclonal antibody (Table 1) for 30–60 min.; rabbit anti-mouse immunoglobulin antibody 1/20 (Dako) for 30 min.; mouse PAP complex 1/200 (Dako) for 30 min.; and diaminobenzidine. Between each step, the sections were washed with phosphate buffered saline (PBS). Finally, they were counterstained with hematoxylin.

For each section, a negative control was performed by replacing the MoAb with PBS. For each series of slides, a positive control consisting of bone marrow with known metastases was used. Suppression of endogenous peroxidase was observed in each series.

Monoclonal antibodies

A panel of five monoclonal antibodies: KL1 [15], CAM5–2 [16], AE1-AE3 [17], EMA [18], and HMFG2 [19] was used (Table 1).

In known BM metastases by conventional histology, IHC gave the following reactions:

- KL1 intensely stained the cytoplasm of all apparent breast carcinoma cells, whether well or poorly differentiated, either isolated or in cords.
- CAM5–2 strongly stained the cytoplasm in many of the carcinoma cells, but apparently not all tumor cells were stained.

Table 1. MoAbs: Reactivities and conditions of use

MoAb	From	Recognized antigen	Dilution
KL1	Immunotech	CK 55 kd	1/150
CAM5–2	Becton-Dickinson	CK 39, 43, 50 kd	not dilut.
AE1-AE3	Hybritech	CK 30, 56.5, 58, 65–67 kd	1/20
EMA	Dako	EMA	1/10
HMFG2	Merck	EMA	1/10

CK: cytokeratin; EMA: epithelial membrane antigen.

- AE1-AE3 were variable in the staining of tumor cell cytoplasm, with not all tumor cells staining.
- EMA and HMFG2 stained only some tumor cells. The positivity was on the cell membrane and occasionally on the cytoplasm. A few cells were stained more intensely; however, apparently certain tumor cells were negative with EMA and HMFG2.

The same results were obtained with the 5 older BM biopsies and 7 recent BM biopsies. Antigen reactivity therefore did not appear to decrease with the BM biopsies preserved in paraffin for a long time.

In no cases with any of the MoAbs were hematopoietic cells positive. Except for the KL1 antibody, the others appeared to be unreliable in the staining of poorly differentiated carcinomas.

Because KL1 MoAb appeared to be the most sensitive, with the others offering no increase in sensitivity, this MoAb was therefore selected to be used alone in all further sections.

Table 2. Clinical and pathological characteristics of group B patients (n = 48)

Characteristics	No of patients
Clinical:	
T stage	
T0–T2	32
T3–T4	16
Inflammatory signs	
Absent*	40
Present	8
Pathological:	
Pathologic tumor size	
<2 cm	14
≥ 2 cm	23
unknown	11
Histological nodal status	
N–	11
N+	26
Unknown	11

* One case with rapid growth (subjective tumor doubling in 6 months).

BM negative by conventional examination

Patients

93 Patients had a negative BM biopsy by conventional techniques and were divided into two groups.

Group A: 45 patients had a BM biopsy performed 6 months to six years after initial diagnosis and therapy (mastectomy and/or radiotherapy and/or chemotherapy) when there was clinical suspicion of bone marrow involvement. These biopsies were obtained between November 1986 and April 1987.

Group B: 48 patients had a BM biopsy performed at initial diagnosis of breast carcinoma. For 39, the biopsy was performed from the anterior superior iliac spine under general anesthetic at the time of the tumorectomy or mastectomy. For the remaining non-operable patients, the BM biopsy was performed from the posterior superior iliac spine under local anesthetic at the time of breast biopsy. Table 2 describes patient characteristics according to the UICC classification [20]. Thirty-one BM biopsies in Group B were performed between 1971–1972 in a study to evaluate the value of 'blind' bone marrow biopsies stained by conventional methods in patients with operable breast carcinoma. This was to give a proper surveillance period if the study proved positive. The 17 other BM biopsies were obtained between November 1986 and April 1987. In this recent series, bone marrow aspirations were not performed simultaneously because they were not routinely done in the older study groups.

Immunohistological method

The method described in preliminary study was used. Only monoclonal antibody KL1 was tested with all BM biopsies.

Results

Group A: In 44/45 cases no positive staining was seen with KL1. One case was positive with KL1 and represented metastatic disease missed by conventional staining.

Group B: No positive results were seen with KL1 staining among the 48 cases.

Discussion

Immunohistochemical techniques allowed the identification of bone marrow metastases in only one of 93 cases negative by standard staining procedures.

As shown by our control cases, immunoreactivity of carcinoma cells is not apparently altered by decalcification techniques. KL1 appears to be the most useful of the MoAbs tested, with an intense positive cytoplasmic reaction in the majority of cells regardless of differentiation, and is more sensitive than CAM5–2 and AE1-AE3. These three anticytokeratin MoAbs apparently stain more intensely than the anti-EMA MoAbs (EMA or HMFG2), but all, except KL1, suffer from limited sensitivity, being unreliable in more undifferentiated tumors. KL1 used by itself appears to be sufficiently sensitive to detect tumor cells and it is doubtful that an expanded antibody panel adds anything but cost [21]. It has the additional advantage of being commercially available and considerably less expensive than some of the other MoAbs. Therefore, KL1 alone was the only MoAb used for all cases.

In the high risk group (A), IHC techniques revealed only one case of BM metastasis not shown by conventional techniques. Here, however, the biopsy did show fibrosis, and hence the diagnosis may have been suspected. Metastases were proven by conventional techniques three months later.

In the relatively low risk group (B), no positive cases were seen among the 48 patients, and our results therefore do not agree with those of others [10, 22, 23], where the use of MoAb IHC techniques permitted the identification of BM metastasis at the time of initial diagnosis. In those studies, using either a rabbit polyclonal serum anti-EMA or a monoclonal anti-EMA antibody to stain multiple bone marrow aspiration preparations, 25–30% of cases were shown to have metastatic cells. However, in only one of those studies [21] had a study previously been performed by conventional techniques to show the increased efficiency of IHC techniques. In another positive study, cell suspensions from bone marrow biopsies, after involved preparation, were stained with another MoAb, MBrl, which apparently recognizes a breast carcinoma associated antigen [24].

Our patient population did not appear to be very different from a positive reported series [10], with an equal number of T0–T2 lesions (67% vs 72%) and similar pathologic tumor sizes (29% vs 35% tumors less than 2 cm). Several reasons may be postulated for these discordant results:

Firstly, in our series, the anterior superior iliac spine was the site of many of our biopsies. Although by BM biopsy the exact iliac site does not appear to be important [25], by aspiration the majority of metastatic cells are found in the posterior superior iliac spine [26]. Secondly, our technique of one biopsy site differs from that of multiple aspiration sites. The latter allows to sample more marrow, and perhaps to detect more metastases, their number increasing with the number of sites sampled [26]. Thirdly, the antibodies employed in the other series were directed against EMA, of which the polyclonal serum is known to stain certain hematopoietic cells, especially plasmacytes [26, 27], and the MoAb stains some immature erythroblasts as well as certain lymphoid tumor cells [28]. These non-specific reactions may make interpretation difficult, and in our opinion, a MoAb such as KL1 with greater specificity in this context and excellent sensitivity appears superior. In a recent study, the presence of cells in bone marrow aspirations stained with polyclonal anti-EMA serum was not found on successive BM aspirations and did not correlate with the development of overt metastases. For the authors, 'This (immunocytochemical) technique is currently insufficiently sensitive to monitor adjuvant accurately therapy in breast cancer'.

Although it appears logical that IHC techniques would complement and augment the search for BM micrometastases afforded by conventional histology, we have not found this to be the case in our series of BM biopsies. Furthermore, no metastases were observed in the patient group characterized as M_0 by conventional procedures at time of initial

therapy. If the detection of metastatic cells in the bone marrow is to be useful for clinical use, more complex procedures are probably necessary than simple bone marrow biopsy stained with IHC techniques.

Acknowledgements

This work was supported by Clinical Research Grant of Institut Gustave-Roussy 87 D16 and Association pour le développement de la Recherche sur le Cancer (ARC). The technical assistance of Mr Y. Lecluse is gratefully acknowledged.

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