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Presence and possible significance of immunocytochemically demonstrable metallothionein over-expression in primary invasive ductal carcinoma of the breast

K.W. Schmid[1], I.O. Ellis[2], Julia M.W. Gee[3], Barbara M. Darke[4], Wendy E. Lees[4], J. Kay[4], A. Cryer[4], J.M. Stark[5], A. Hittmair[6], D. Öfner[7], Martina Dünser[7], R. Margreiter[7], G. Daxenbichler[8], R.I. Nicholson[3], B. Bier[1], W. Böcker[1], B. Jasani[9]

[1] Department of Pathology, University of Münster, Münster, Germany
[2] Department of Pathology, Nottingham City Hospital, Nottingham, UK
[3] Breast Cancer Unit, Tenovus Institute, Cardiff, UK
[4] Department of Biochemistry, University of Wales College of Cardiff, Cardiff, UK
[5] Department of Medical Microbiology, University of Wales College of Medicine, Cardiff, UK
[6] Department of Pathology, University of Innsbruck, Innsbruck, Austria
[7] I. Department of Surgery, University of Innsbruck, Innsbruck, Austria
[8] Biochemical Laboratory of the Clinic of Gynaecology, University of Innsbruck, Innsbruck, Austria
[9] Department of Pathology, University of Wales College of Medicine, Cardiff, UK

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Abstract. Metallothioneins (MTs) are ubiquitous low-molecular-weight proteins with a high affinity for heavy metal ions such as zinc, copper and cadmium. MT over-expression has been associated with resistance against anticancer drugs. In the present study we investigated 86 cases (45 cases of tumour category pT1 and 41 of category pT2) of routinely fixed and paraffin-embedded primary breast carcinomas immunohistochemically with a monoclonal antibody to an epitope of MT shared by its I and II isoforms. Immunohistochemically demonstrated MT over-expression was found in the invasive components of 7 of 32 pT1 and 17 of 28 pT2 invasive ductal carcinomas, whereas all 26 invasive lobular carcinomas gave weak or negative results. Fourteen of 17 pT2 and 2 of 7 pT1 invasive ductal carcinomas with MT over-expression developed metastases during follow-up with poor prognostic outcome. In contrast only 3 of 11 pT2 and none of the 25 pT1 cases without MT over-expression had a poor clinical course ($P<0.001$). It is concluded that MT over-expression is associated with significantly poor prognosis particularly in pT2 invasive ductal breast carcinomas.

Key words: Metallothionein – Breast cancer – Oestrogen receptors – Progesterone receptors – Immunohistochemistry

Correspondence to: K.W. Schmid, Department of Pathology, Domagkstrasse 17, W-4400 Münster, Germany

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Introduction

Metallothioneins (MTs) are ubiquitous low-molecular-weight proteins with a high content (approximately 30%) of cysteine, exhibiting a selective binding affinity for zinc, copper and other group II heavy metal ions. Synthesis of MTs is induced in a variety of tissues by these metal ions as well as by endogenous factors such as glucocorticoids, interferon, interleukin-1 and vitamin D_3.

MTs have the ability to bind to large quantities of metal ions, which implies an intracellular reservoir or sequestration function for essential or potentially toxic ions such as zinc and copper, respectively. Furthermore MTs play an important role in the detoxification of toxic metals such as cadmium (Friberg et al. 1974; Leber and Miya 1976; Nomiyama et al. 1982; Goering and Klaassen 1984) and possibly in the cellular protection against ionizing radiation and alkylating agent cytotoxicity (for a review, see Nath et al. 1988).

Increased expression of MTs has been implicated in drug resistance expressed by cell lines derived from a variety of cancers (Kelley et al. 1988). Decrease in the cytotoxic activity of certain anticancer drugs has been reported as well as an increased resistance in MT-rich cells during exposure to ionizing radiation. However, the mechanism by which MTs contribute to this protection is still unclear. It has been suggested that sequestration of drugs or their metabolites may prevent the reaction of these compounds with the respective intracellular targets. Another capacity of MTs is to scavenge free radicals which may contribute to their radio-protective effects (Thornalley and Vasak 1985).

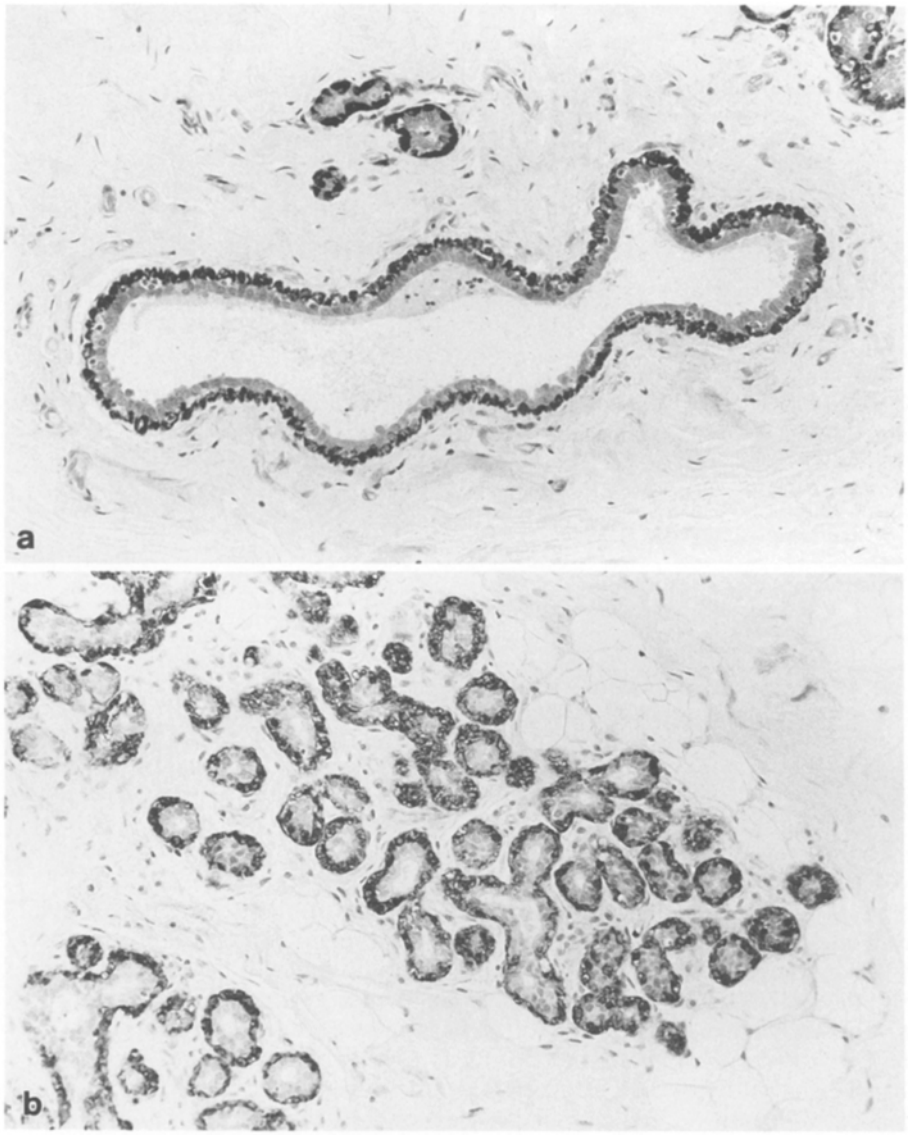

Fig. 1. a Ductal component of normal breast tissue with strong metallothionein (MT) staining of myoepithelial cells. Indirect immunoperoxidase, haematoxylin counterstain, ×100. **b** A strong MT positivity was also present in myoepithelial cells of the lobular component. Indirect immunoperoxidase, haematoxylin counterstain, ×100

In the present study we investigated 86 cases of primary breast cancer immunohistochemically by means of a monoclonal anti-MT (I and II isoforms) antibody and correlated the immunohistochemical findings with the pTNM classification, the clinical outcome, the oestrogen (ER) and progesterone receptor (PR) status, and the histological tumour type of the respective patients.

Materials and methods

Randomly selected and routinely formalin-fixed and paraffin-embedded tissues from 100 primary adenocarcinomas of the breast were reclassified independently by three of the authors (K.W.S., A.H., J.M.W.G.). The staging of the tumours was performed according to the UICC recommendations (TNM-classification; Spiessl et al. 1989) for breast cancer. After reclassification the present series comprised 86 cases including 32 invasive ductal and 13 invasive lobular carcinomas of tumour category pT1 and 28 invasive ductal and 13 invasive lobular carcinomas of tumour category pT2.

Balb/c mice (aged 6–8 weeks) were immunized with 100 μg horse metallothionein (mixture of isoforms obtained from Sigma, product no. 4766) in complete Freund's adjuvant. A second booster injection (100 μg metallothionein/mouse) was given in incomplete Freund's adjuvant. Three days prior to spleen removal, a pre-fusion boost was administered by intravenous injection of metallothionein (100 μg without adjuvant). Fusion of spleenocytes with P2-X63-Ag8.653 mouse myeloma cells was carried out using 40% polyethylene glycol 1500. Cells secreting antibodies to metallothionein were selcted by ELISA using peroxidase-conjugated anti-mouse IgG. Positive cells were cloned by limiting dilution and monoclonal lines were propagated in pristane-primed Balb-c mice. The asictes fluid obtained of these mice was pooled and used without further purification.

The monoclonal anti-MT antibody E9 has been found (Jasani and Elmes 1991) to be immunocytochemically reactive against a conserved epitope shared by I and II isoforms of human, rat and horse MT. The antibody has been used successfully to detect immunoreactive MT in formalin-fixed, paraffin-embedded tissues of rat and human origin (Elmes et al. 1989; Evering et al. 1990; Fuller et al. 1990).

Tissue blocks were cut at a thickness of 4 μm and mounted on chrome-gel-coated glass slides. After dewaxing and rehydrating in a series of alcohols, endogenous peroxidase was blocked with sodium azide, glucose and glucose-oxidase (all obtained from Sigma, Munich, Germany) according to a modified regimen (Hittmair

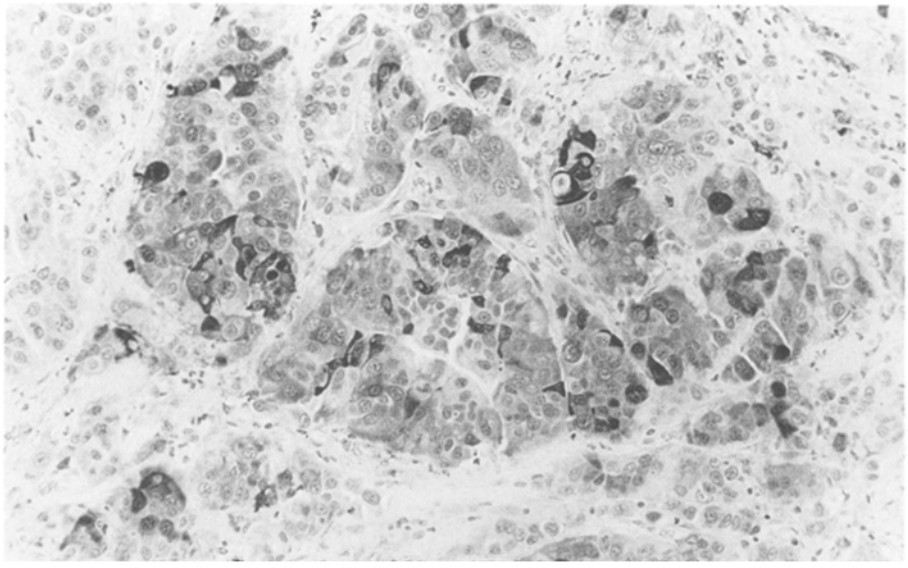

Fig. 2. Weakly to strongly stained tumour cells of an invasive ductal carcinoma. Note MT positivity in the cytoplasm and the nucleus of some tumour cells. Indirect immunoperoxidase, haematoxylin counterstain, ×200

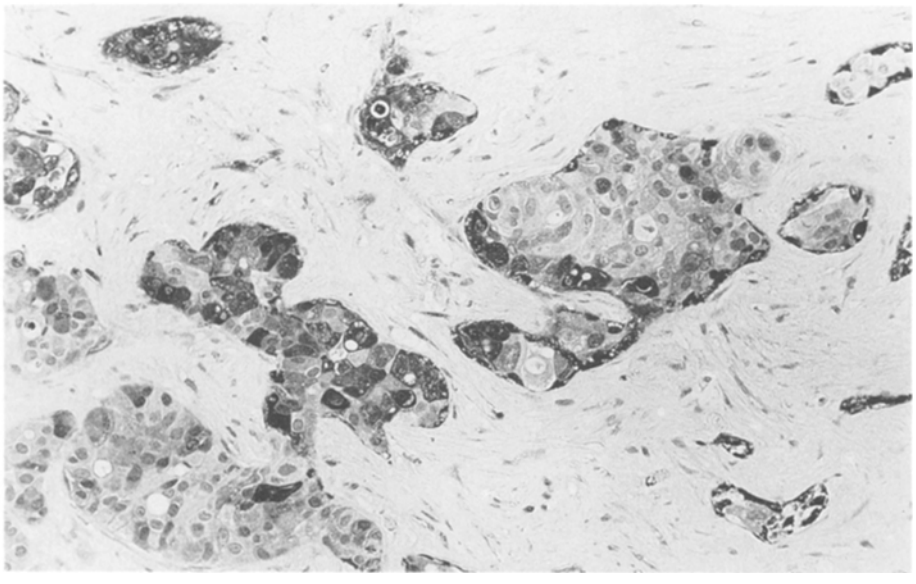

Fig. 3. Invasive ductal carcinoma with a high proportion of tumour cells being strongly MT-positive. Note nuclear staining in otherwise negative or only weakly MT-positive tumour cells. Indirect immunoperoxidase, haematoxylin counterstain, ×200

and Schmid 1989) originally described by Andrew and Jasani (1987). The monoclonal primary MT antibody was applied in a humidified chamber overnight at 4° C, (dilution 1:20,000 in 0.6% bovine serum albumin) followed by a peroxidase-conjugated rabbit anti-mouse antibody (1:150; Dako, Copenhagen, Denmark) for 45 min at room temperature. The enzyme reaction was developed in a freshly made solution containing diaminobenzidine (0.5 mg/ml; Sigma) and 0.01% hydrogen peroxide (Merck, Heidelberg, Germany) for 5–7 min. Finally the sections were counterstained with haematoxylin, dehydrated, cleared with xylene and mounted with Entellan (Merck).

Patients were divided into two groups. Group I consisted of patients which developed tumour recurrence and/or metastases during the follow-up period. In the second group, patients remained tumour free at the time of the assessment for at least 60 months.

For ER and PR immunohistochemical assays (ER-ICA, PR-ICA), dextran-coated charcoal (DCC), and enzyme immuno-assays (ER-EIA, PR-EIA) were performed according to the recommendations of the E.O.R.T.C. receptor study group (E.O.R.T.C. Breast Cancer Cooperative Group 1980). These were performed on frozen tumour tissue material, matched with respect to the tissue taken for histology. The lower cut-off point for ER-ICA and PR-ICA was less than 5% positive nuclei, and those for DCC, ER-EIA and PR-EIA were 10 (ER) and 25 (PR) fmol/mg, respectively.

Results

In normal breast tissue MT expression was found in myoepithelial cells and only occasionally in ductal cells. No MT could be demonstrated within the alveolar component of normal breast tissue (Fig. 1a, b).

Evaluation of the immunohistochemical MT expression was confined to the invasive parts of the respective breast carcinoma specimens and was divided as follows: complete lack of MT-positive cells (−), scattered single tumour cells MT-positive (+), focal moderate to strong MT positivity (++; Fig. 2), and more than 50% of tumour cells MT-positive (+++; Fig. 3). MT positivity

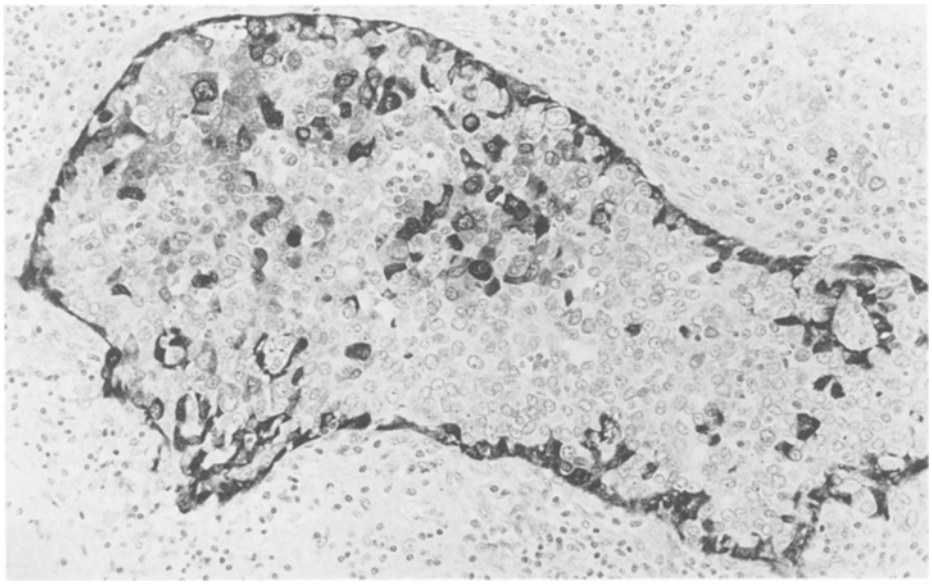

Fig. 4. Non-invasive ductal component of an otherwise invasive lobular breast carcinoma with a rim of MT-positive tumour cells and some positive cells in the centre of the tumour cell formation. Indirect immunoperoxidase, haematoxylin counterstain, ×200

Table 1. Metallothionein (MT) expression in human breast cancer cases of tumour categories pT1 and pT2

Histological classification pT classification	Clinical course	*n*	−	+	++	+++
pT1						
Invasive ductal carcinoma	Tumour free	30	15	10	3	2
	Metastases	2	–	–	1	1
Invasive lobular carcinoma	Tumour free	13	7	6	–	–
	Metastases	0	–	–	–	–
pT2						
Invasive ductal carcinoma	Tumour free	11	4	4	1	2
	Metastases	17	2	1	3	11
Invasive lobular carcinoma	Tumour free	5	3	2	–	–
	Metastases	8	3	5	–	–

was demonstrable either in the cytoplasm or the nucleus or in both. Tumours with ++ and +++ level of staining were classified as showing MT over-expression. In all cases the division between MT-positive scattered single tumour cells and focal staining was a clear-cut one.

A summary of the immunohistochemical findings with regard to MT over-expression is given in Table 1. MT over-expression was found in 5 of 32 pT1 invasive ductal carcinomas (Figs. 2, 3). Two of these 5 cases with MT over-expression developed metastases during follow-up. Amongst the 28 pT2 invasive ductal carcinomas 3 out of 11 cases with favourable outcome contained a considerable number of MT-positive tumour cells whereas only 3 out of 17 invasive ductal carcinomas with subsequent development of metastases showed less than 5% MT-positive tumour cells. Two of the 3 patients who were MT-negative but developed metastases during follow-up did not receive any kind of treatment (cases 5 and 11). Amongst the 3 cases which, though MT-positive, have been tumour free for 69 (case 12), 84 (case 16), and 66 months (case 28) respectively, cases 12 and 16 received an anti-oestrogen therapy and case 12 irradiation therapy in addition.

Invasive lobular carcinomas (pT1 and pT2) occasionally showed a few MT-positive tumour cells (14/26) or were completely MT-negative. Some invasive lobular carcinomas contained non-invasive ductal components which usually exhibited in the periphery a typical rim of MT-positive cells (Fig. 4).

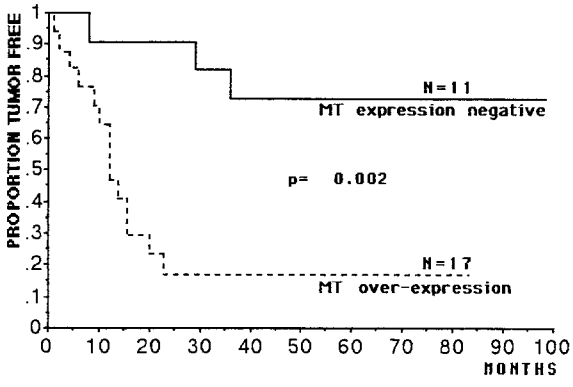

Fig. 5. Kaplan-Meier curve showing the tumour-free interval of patients with pT2 invasive ductal carcinomas in regard to MT over-expression

Table 2. Clinical data and MT expression in the 28 invasive ductal carcinomas of tumour category pT2

No.	Age (years)	pTNM	ER	PR	AET	IRR	PCTH	Clinical course (months)	MT status
1.	47	T2N1	−	−	−	−	+	Tumour free (78)	−
2.	60	T2N0	+	+	+	+	−	Tumour free (78)	−
3.	47	T2N2	+	+	+	+	+	Tumour free (66)	−
4	73	T2N2	+	+	+	+	+	Metastases (8)	−
5.	70	T2N1	+	+	−	−	−	Metastases (36)	−
6.	69	T2N0	+	+	−	−	−	Tumour free (76)	−
7.	34	T2N2	+	−	+	+	+	Tumour free (93)	+
8.	36	T2N0	−	−	−	+	−	Tumour free (74)	+
9.	54	T2N0	+	+	+	−	−	Tumour free (85)	+
10.	46	T2N0	+	−	−	−	−	Tumour free (98)	+
11.	48	T2N0	+	−	−	−	−	Metastases (29)	+
12.	70	T2N2	+	+	+	+	+	Metastases (16)	++
13.	44	T2N2	−	−	−	−	+	Metastases (10)	++
14.	56	T2N2	+	+	+	+	−	Tumour free (69)	++
15.	52	T2N0	−	−	−	−	−	Metastases (4)	++
16.	72	T2N1	−	−	−	−	+	Metastases (12)	+++
17.	60	T2N1	−	−	−	−	+	Metastases (1)	+++
18.	54	T2N0	+	+	+	−	−	Metastases (12)	+++
19.	58	T2N1	+	−	+	−	+	Tumour free (84)	+++
20.	59	T2N2	−	−	−	−	+	Metastases (2)	+++
21.	59	T2N1	+	+	+	−	+	Metastases (20)	+++
22.	33	T2N1	+	+	+	−	+	Metastases (23)	+++
23.	63	T2N1	−	−	+	−	+	Metastases (16)	+++
24.	40	T2N1	−	−	−	−	+	Metastases (14)	+++
25.	53	T2N1	+	−	−	+	−	Metastases (9)	+++
26.	66	T2N1	+	−	−	+	−	Metastases (6)	+++
27.	56	T2N1	+	−	−	+	−	Metastases (12)	+++
28.	67	T2N1	+	−	−	+	−	Tumour free (66)	+++

ER, Oestrogen receptors; PR, progesterone receptors; AET, anti-oestrogen therapy; IRR, irradiation therapy; PCTH, polychemotherapy

Table 3. MT expression and oestrogen receptor status in invasive ductal carcinomas of tumour category pT2

Receptor status	Clinical course	*n*	−	+	++	+++
Positive	Tumour free	9	3	3	1	2
	Metastases	10	2	1	1	6
Negative	Tumour free	2	1	1	−	−
	Metastases	7	−	−	2	5

Two-way tables significance tests (small sample size, $n \leq 20$): for ER-positive cases not significant, for ER-negative cases $P=0.002$, $\chi^2=9.35$

Table 4. MT expression and progesterone receptor status in invasive ductal carcinomas of tumour category pT2

Receptor status	Clinical course	*n*	−	+	++	+++
Positive	Tumour free	5	3	1	1	−
	Metastases	6	2	−	1	3
Negative	Tumour free	6	1	3	−	2
	Metastases	11	−	1	2	8

Two-ways tables significance test (small sample size $n \leq 20$): for PR-positive cases not significant, for PR-negative cases $P=0.01$, $\chi^2=6.26$

Table 5. Prognostic factors of 28 cases of pT2 invasive ductal breast carcinoma: a univariate approach to tumour-free intervals in patients potentially curable by surgery (compare also Fig. 5)

	df	Univariate chi-squares for the log rank test	*P*
MT over-expression (negative vs over-expression)	1	9.51	0.002
ER status (negative vs positive)	1	3.63	0.05
pTNM (pT2N0 vs pT2N1 and pT2N2)	1	2.17	NS
PR status (negative vs positive)	1	0.97	NS
Age (<55 vs >55 years)	1	0.52	NS

df, Degree of freedom; MT, metallothionein; ER, oestrogen receptors; PR, progesterone receptors; NS, not significant

The clinical data (ER status, PR status, various kinds of treatment, development of metastases, tumour-free survival interval) for the 28 patients with invasive ductal carcinomas of tumour category pT2 and their corresponding MT expression are given in Table 2. The tumour-free interval in respect to the level of MT over-expression in these cases is shown in Fig. 5.

A comparison of the level of MT expression, clinical outcome and ER status in the 28 pT2 invasive ductal

carcinomas is given in Table 3. A similar set of data are given in relation to the PR status in Table 4. Evaluation of MT over-expression, ER and PR status, and tumour stage in relation to the clinical outcome is given in Table 5. Statistical analysis was performed using the Systat statistical package (Systat Inc., Evanstone, Ill., USA). All values are given as means. The functions for the tumour-free interval were estimated by the product-limit method (Kaplan and Meier 1958); for comparison of the respective curves the log rank test (Mantel-Haenszel Test; Kalbfleisch and Prendice 1980) was used. Variables were considered either as continuous or categorical. TNM analysis was performed using a set of dummy variables. Each of the prognostic factors was examined individually by univariate life-table analysis (Kalbfleisch and Prentice 1980).

Discussion

Our results indicate that over-expression of MT in invasive ductal carcinomas of the breast is associated with poor prognosis which seems not to be related to therapy. Possible association of MT expression with drug resistance and ER and PR status has not been examined systematically. The detection of MTs in tissue by histological means has been based on histochemical techniques until fairly recently (Jasani and Elmes 1991). In the present study we have taken advantage of the newly developed immunohistochemical method effective for MT detection in routinely formalin-fixed, paraffin-embedded tissue sections (Evering et al. 1990; Fuller et al. 1990).

Although the involvement of MTs in conferring resistance to anticancer agents remains controversial (Kelley et al. 1988) the results of our retrospective immunohistochemical study on breast cancer cases are supportive of the theory that MTs may play an important role in defending cells against anticancer drugs. The development of the so-called multi-drug resistance in human breast cancer cells has been associated with over-expression of P-glycoprotein (Beck et al. 1979; Juliano and Ling 1976; Kartner et al. 1983; Peterson et al. 1983), loss of hormone sensitivity and ER (Vickers et al. 1988), and alterations in the activities of detoxication enzymes such as glutathione S-transferase (Moscow et al. 1988) and MTs.

MT over-expression in the pT2 invasive ductal carcinomas of our series was significantly correlated with the development of metastases. Two of the 3 patients with over-expression of MT developed metastases and did not receive any kind of cancer specific treatment. It is interesting to see that in these 3 cases metastases developed after a longer tumour-free interval than in any other case with metastases. However, all the cases with MT over-expression which are tumour free 66, 69, and 84 months after surgery were ER positive. We may speculate that the anti-oestrogen therapy applied in 2 of these 3 cases was successful.

MT over-expression was found in 7 of 32 pT1 invasive ductal carcinomas. There were only 2 pT1 cases with development of metastases during follow-up, but both exhibited MT over-expression. A strong MT positivity was seen in the periphery of in situ components (myoepithelial cells) of 24 pT1 cases whereas only 5 of 24 cases exhibited MT over-expression in the invasive parts of the respective tumours.

MT over-expression has been shown in various carcinoma cell lines resistant to certain anticancer drugs (Andrews et al. 1987; Kelley et al. 1988; Webber et al. 1988). Whilst over-expression of MTs is inducible by a variety of endogenous and exogenous mechanisms involving heavy metal ions, glucocorticoid hormones, interferon, interleukin-1 and vitamin D_3 as well as by increased cell activity (Hamer 1986; Karasawa et al. 1987; Nartey et al. 1987) the recently published induction of MT gene expression by progesterone (Slater et al. 1988) points to yet another regulatory mechanism for MT synthesis. However, the PR status of the cases of this study did not show an obvious correlation with MT expression and prognosis (compare Tables 4 and 5).

MT over-expression was not significantly correlated with prognosis in pT2 cases with positive ER status. However, there was a significant relationship in ER-negative cases (Table 3). Thus it seems that MT over-expression in ER-negative breast cancer cases may indicate poor clinical outcome.

Our results demonstrate that MT over-expression may already be present in breast carcinomas at the time of operation. Cancer cell lines with increased MT expression have been shown to be resistant to clinically important cancer chemotherapeutic agents (Kelley et al. 1988). Additionally, cells with acquired resistance to anticancer drugs fequently show an increase in MTs and over-express MT mRNA. MT over-expression in metastases of cases with MT-negative primary tumours would suggest such a mechanism in vivo. Although other mechanisms of resistance to anticancer drugs are most likely to exist (Frei et al. 1985; Scanlon and Kashani-Sabet 1985; Teicher et al. 1986, 1987) MTs may play an outstanding role in drug resistance. Therapeutic agents such as interferon and steroids induce MT expression and trigger drug resistance. Nevertheless it has been shown in a prostate cancer cell line that lowering of intracellular MTs can enhance the sensitivity of these cells to Adriamycin (Webber et al. 1988). Agents capable of decreasing MT expression may help to reduce or even prevent MT activity as a mechanism of resistance to anticancer drugs.

To what extent the lack of MT expression plays a significant role in the prognosis of invasive lobular carcinomas remains debatable. Our finding that primary invasive lobular carcinomas almost completely lack MT-expressing tumour cells may not necessarily imply that these tumours cannot acquire MT expression during tumour progression and/or treatment.

In conclusion, MT over-expression in pT2 invasive ductal breast carcinoma, demonstrated immunohistochemically in the primary tumours, seems to be significantly associated with poor prognosis. However, further investigations are required to elucidate the complex mechanisms contributing to MT over-expression, on the

one hand, and its possible involvement in promotion of poor prognostic outcome, on the other. Large-scale investigations are in progress to compare special subtypes of invasive breast cancer with MT over-expression.

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