Virchows Archiv A Pathol Anat (1993) 423:215–219

Virchows Archiv A
Pathological Anatomy and Histopathology

Localization of metallothionein in breast carcinomas. An immunohistochemical study

Manuel Fresno[1], Weiyu Wu[2], Jose M. Rodriguez[3], Mehrdad Nadji[2]

[1] Department of Pathology, University of Oviedo, Hospital Covadonga, Oviedo, Spain
[2] Department of Pathology, University of Miami, Jackson Memorial Medical Center, Miami, Florida, USA
[3] Department of Gynecology, University of Oviedo, Hospital Covadonga, Oviedo, Spain

Received April 5, 1993 / Received after revision June 12, 1993 / Accepted June 14, 1993

Abstract. Metallothionein (MT) is a cysteine-rich, low molecular weight protein that binds zinc, copper, and cadmium. It is present in a number of normal cells including hepatocytes particularly during fetal and early postnatal life. It has been suggested that developmental profile of MT is similar to other oncofetal gene products and hence, it could be used as a marker for aggressive tumour behaviour. In order to test that hypothesis, we used a monoclonal antibody to MT and immunohistochemically evaluated formalin-fixed, paraffin-embedded tissues from 79 breast carcinomas. In non-neoplastic breast tissue, a strong nuclear and cytoplasmic staining was observed in myoepithelial cells. Positive staining for MT was present in 35 (44%) of breast carcinomas. In most positive cases, nuclear, or both nuclear and cytoplasmic staining was seen. All positive tumours were invasive ductal carcinomas, including a medullary and a metaplastic carcinoma. None of the mucinous, lobular, or intraductal papillary carcinomas reacted for MT. A statistically significant association was found between MT immunostaining and histological grade ($P<0.01$) as well as with nuclear grade ($P<0.01$). We also observed an inverse relationship between MT staining and oestrogen receptor content of tumours ($P<0.01$). Similarly, a statistically significant association was found between moderate and strong MT immunostainig and decreased overall survival and shorter disease-free survival ($P<0.01$). MT immunostaining was also predective of a worse prognosis in the subgroup of lymph node negative ($P<0.001$) and oestrogen receptor negative patients ($P<0.01$). No statistically significant association was found between MT staining and size of tumour or the presence of lymph node metastasis. We conclude that MT staining may be a useful marker of less differentiated and more aggressive carcinomas of the breast.

Key words: Metallothionein – Breast cancer – Immunohistochemistry

Correspondence to: M. Fresno, Department of Pathology, Hospital Covadonga, c) Celestino Villamil s/n, E–Oviedo 33006, Spain

Supported by FISS Grant 90–5146. Presented at the XIX International Congress of the International Academy of Pathology, Madrid 18–23 October 1992.

Introduction

Metallothioneins (MTs) are a group of intracellular metalloproteins of low molecular weight (6–7 kDa) with a high content of cysteinyl residues . MTs have a high affinity for metal ions such a zinc, copper, cadmium, silver, platinum and mercury. (Kagi et al. 1974; Cherian and Goyer 1978; Kagi and Nordberg 1979). During gestation and early postnatal period high levels of endogenous MT bound to copper and zinc have been observed in mammalian liver with levels declining to very low concentrations in adult life (Bremmer et al. 1977; Ryden and Deutsh 1978; Benerjee et al. 1982).

MTs are considered to be involved in various physiological processes, especially storage of essential metals (Webb and Cain 1982; Panemangalore et al. 1983). The presence of MT in the developing liver and the endodermal yolk sac of mice has suggested that developmental profile of MT is similar to other oncofetal gene products such as alpha-fetoprotein and that it has potential use as tumour marker. MT has been localized in human testicular embryonal carcinomas as well as in thyroid tumours (Kontozoglou et al. 1987; Nartey et al. 1987). Recently, (Schimd et al. 1993) MT has been demonstrated immunohistochemically in breast carcinoma where its over-expression was associated with significantly poor prognosis. The production of MT by tumour cells has been proposed as a major factor contributing to the resistance of those tumours to metal-containing chemotherapeutic compounds (Eastman and Richon 1985).

In this study we have explored the immunohistochemical localization of MT in breast carcinomas and its possible correlation with morphological characteris-

tics of the tumours, as well as with oestrogen receptor content and patient prognosis.

Materials and methods

Formalin-fixed, paraffin-embedded blocks of 79 breast carcinomas from the Hospital Covadonga (University of Oviedo, Spain) were used in this study. The tumours were classified according to the WHO histological typing. They included 63 invasive ductal carcinomas of no special type, 5 medullary carcinomas, 4 mucinous carcinomas, 4 invasive lobular carcinomas and 1 of each intraductal and metaplastic carcinoma, and Paget's disease. Histological grades of tumours were scored according to Bloom and Richardson (1957). The results of oestrogen receptor content as determined with dextran-coated charcoal technique were available on all invasive tumours. Values of more than 10 fmol/mg protein were considered positive.

We used a specific anti-metallothionein mouse monoclonal antibody (DAKO, Carpenteria, Calif., USA) at dilution 1:50 and a standard biotin avidin peroxidase complex technique. Diaminobenzidine-hydrogen peroxidase was employed as the chromogen and a light haematoxylin counterstain was used. We used in all cases a positive control, usually a known positive breast tumour. Myoepithelial cells, were a usefull positive internal control. Negative controls consisted of substitution or primary antibody by PBS; all others steps were followed unchanged.

Immunoperoxidase reactivity was scored based on the percentage of tumour cells stained: 0= no staining at all; 1+ = scattered single stained cells, less than 10%; 2+ = between 10–50% of cells stained; 3+ = more than 50% of cells stained.

The relationship between immunohistochemical staining and morphological variables were evaluated by means of univariate Chi-square test for association. Analyses of disease-free and overall survival were performed with use of the Kaplan–Meier method (1958). Test of differences between curves were made with the log-rank tests (Peto et al. 1977). For survival study, cases were divided into two groups: MT over-expression cases (moderate and strongly stained cases) and negative, weak and focally stained cases (1+ staining intensity).

Results

MT immunostaining was found in 35 of 79 tumours (44%). Seven cases were scored 3+ (more than 50% of cells stained)), 9 cases 2+ (between 10–50%) and 19 cases 1+ (less than 10% of cells stained). Tumors with 2+ (moderate) or 3+ (strong) staining intensity were classified as showing MT over-expression. The division between scatered single stained cells (1+) and cases with moderate staining was clear-cut. Staining was, both cytoplasmic and nuclear in most cases, (Fig.1). Nuclear staining alone was observed in 6 cases; all these were scored 1+. The staining intensity and distribution of stained cells was heterogeneous within the same tumour. Stained and unstained cells or cell clusters were present side by side. Apart from cases that showed only nuclear staining intensity was moderate or strong. There was no correlation between staining intensity and percentage of staining cells. We observed strong cytoplasmic and nuclear staining in tumours that had only scattered reactive cells (Fig. 2). Although some cells showed only cytoplasmic staining, in most of the cells both cytoplasmic and nuclear staining was present. We also observed immunostaining in myoepithelial cells (Fig. 3) and in fibroblasts and stromal histiocytes (Fig. 4). Only occasional weak nuclear staining was seen in normal duct cells. The distribu-

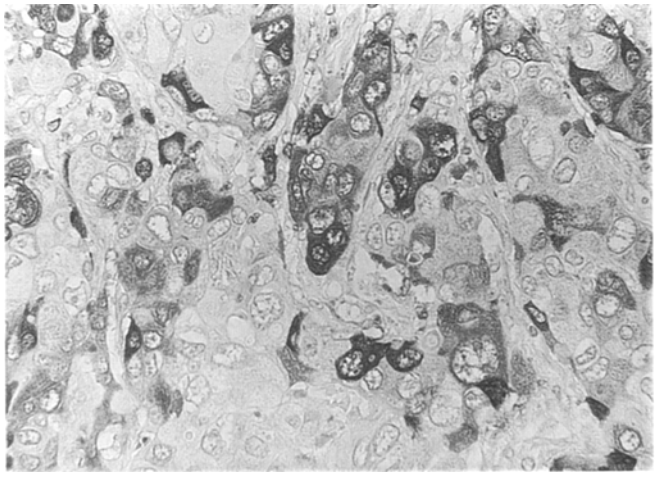

Fig. 1. Immunohistochemical localization of metallothionein (MT) in the nucleus and cytoplasm of tumour cells. × 400

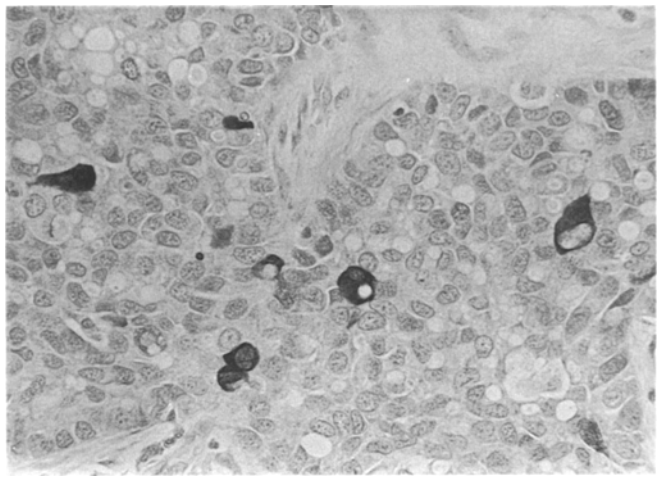

Fig. 2. Strong cytoplasmic and nuclear immunostaining in scattered tumour cells. × 200

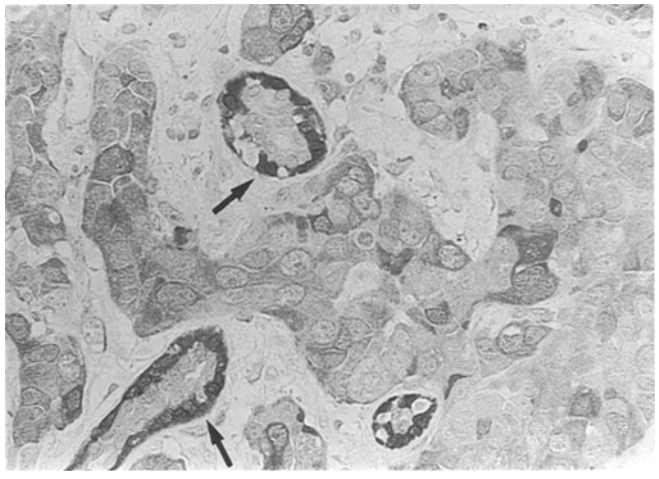

Fig. 3. MT immunostaining in tumour and myoepithelial cells *(arrow)*. No staining seen in other non-neoplastic cells. × 400

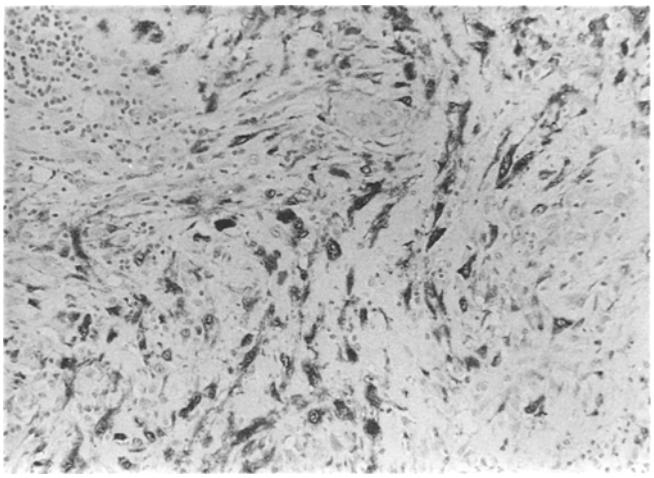

Fig. 4. Positive immunostaining in fibroblastic and stromal histiocytes. × 100

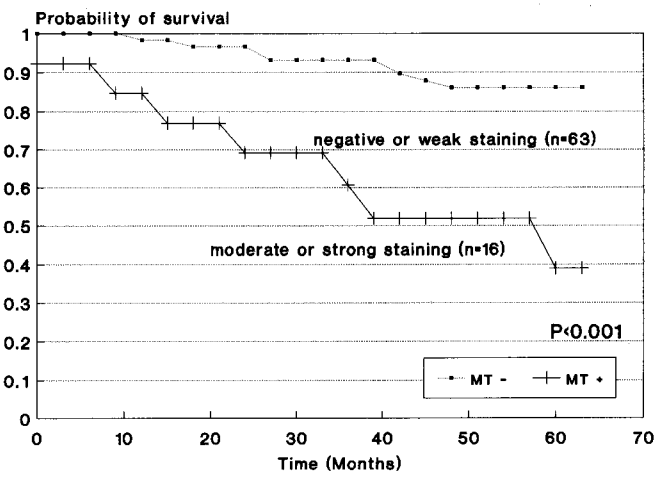

Fig. 5. Overall survival of patients relative to immunohistochemical staining for MT

Table 1. Immunohistochemical localization of metallothionein (MT) in breast tumours

Histological type of carcinoma (WHO)	Number of tumours	Positive	Score		
			3+	2+	1+
Invasive ductal	63	32	6	8	18
Medullary	5	2		1	1
Mucinous	4	0			
Lobular	4	0			
Paget's disease	1	0			
Intraductal	1	0			
Metaplastic	1	1	1		

tion of MT immunostaining according to histological types is shown in Table 1.

All cases that scored 3+ or 2+ were invasive ductal carcinomas including one medullary carcinoma and one metaplastic carcinoma. All mucinous and lobular carcinomas were negative.

The association between MT immunostaining and morphologic parameters with prognostic significance is shown in Table 2.

We observed a statistically significative association between MT immunostaining and nuclear and histological grade ($P<0.01$). High histological and nuclear grade

Table 2. Association between MT immunostaining, morphological parameters and oestrogen receptor (ER) status

	MT-immunostainig			Score		
	Negative	Positive	*P* value[a]	Negative	1+	2+	3+
Lymph node status							
Metastasis	16	18	NS	16	12	4	2
No metastasis	27	16		27	7	5	4
Nuclear grade							
I	12	2	$P<0.01$	12	0	0	0
II	19	10		19	9	1	0
III	13	23		13	10	7	6
Histological grade							
I	13	3	$P<0.01$	13	2	1	0
II	22	13		22	9	2	2
III	9	19		9	8	6	5
Size							
Smaller than 2 cm	16	11	NS	15	7	4	2
Between 2–5 cm	26	20		27	10	5	4
More than 5 cm	2	3		2	2	0	1
ER status							
Positive	35	17	$P<0.01$	35	11	4	2
Negative	9	18		9	8	5	5

NS, Not significant

[a] Chi-square was calculated only with positive and negative cases

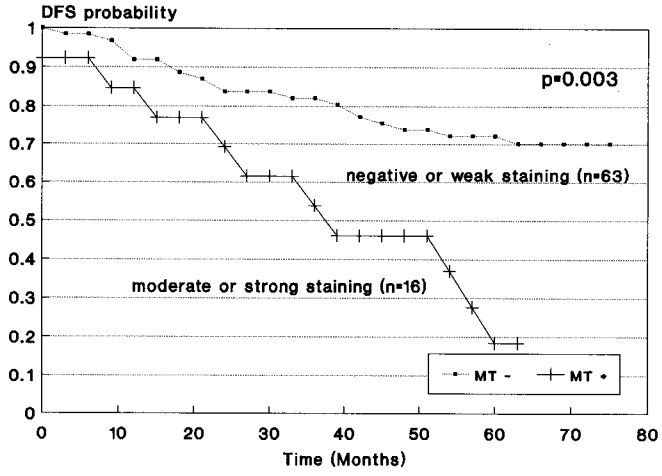

Fig. 6. Disease-free survival of patients relative to immunohistochemical staining for MT

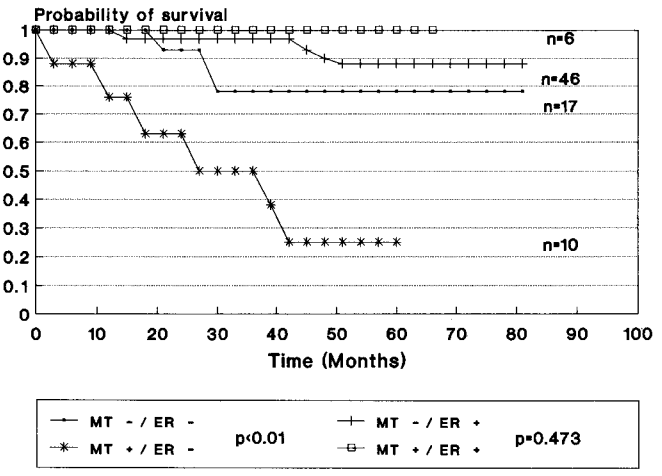

Fig. 9. Overall survival of patients with ER negative and ER positive tumours relative to MT immunostaining. Weakly stained cases were considered negative

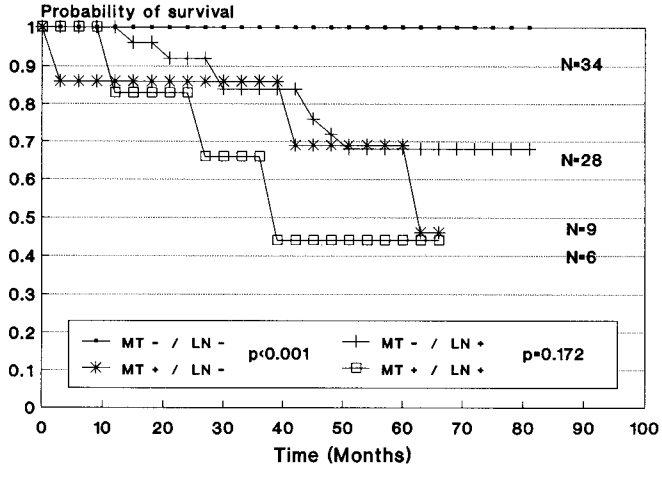

Fig. 7. Overall survival of patients with node negative (LN–) and node positive (LN+) tumours relative to MT immunostaining. Weakly-stained cases were considered negative

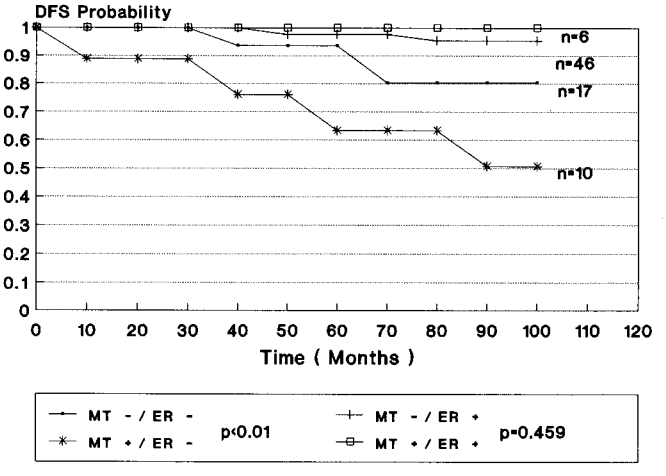

Fig. 10. Disease-free survival of patients with ER negative and ER positive tumours relative to MT immunostaining. Weakly stained cases were considered negative

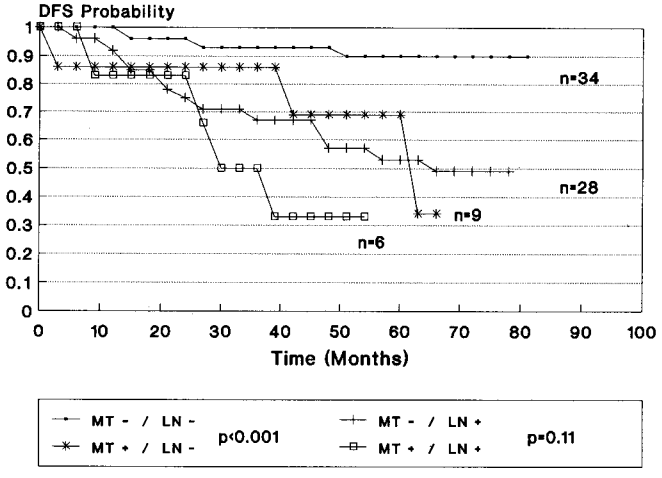

Fig. 8. Disease-free survival of patients with node negative and node positive tumours relative to MT immunostaining. Weakly stained cases were considered negative

tumours were more frequently MT positive and all cases scored 3+ were nuclear grade III.

We also observed a statistically significant ($P<0.01$) inverse associaton between MT expression and oestrogen receptor content of the tumours (Table 2).

Thirty-five patients had one or more of the axillary lymph nodes involved by cancer. No association was found between lymph node status and MT expression.

Fifteen patients died of the cancer between 1 and 61 months after the initial surgery (average, 30 months). Fourteen patients relapsed between 4 and 79 months (average, 39 months). Forty-six patients were alive at the end of the follow-up between 20 and 79 months after surgery (average, 66 months). Four patients were lost to follow-up.

Figures 5 to 10 shows the association of MT immunostaining with disease-free and overall survival. A statistically significant association was found between moderate and strong MT immunostainig and decreased

overall survival and shorter disease-free survival. MT immunostaining was also predective of a worse prognosis in the subgroups of lymph node negative and oestrogen receptor negative patients.

Discussion

We have demonstrated the presence of MT in breast carcinoma using an immunohistochemical method. Previous studies have demonstrated MT in thyroid and testicular embryonal carcinomas but no correlation was made with tumour differentiation or tumour aggressiveness (Kontozoglou et al. 1987; Nartey et al. 1987). In a recent report, (Schmid et al. 1993), using an immunohistochemical method, MT over-expression was associated with poor prognosis particularly in pT2 invasive ductal carcinomas and oestrogen receptor (ER) negative tumours. As in our results, MT over-expression in ER-negative breast tumours may indicate poor clinical outcome.

Studies in thyroid and testicular tumours have demonstrated a high positivity (100% in testicular embryonal carcinoma and 91% in thyroid) tumours. We have observed 47% positivity mainly in the group of invasive ductal carcinomas. Findings of MT negativity in invasive lobular carcinoma were recently published by Schmid et al. (1993) where most of the stained tumours showed a cytoplasmic and nuclear staining and no cytoplasmic staining was observed in normal breast tissue. An immunohistochemical staining pattern similar to our cases was found in the Schmid report (Schmid et al. 1993) with MT positivity either in the cytoplasm or the nucleus or in both and positivity in myoepithelial cells. In a previous study in thyroid adenomas and carcinomas, nuclear staining was shown in adenomas, whereas in most of carcinomas both nuclear and cytoplasmic MT staining was observed (Nartey et al. 1987). Whether the nuclear or cytoplasmic staining reflect a de-differentiation of the tumour cells with re-expression of previously suppressed oncofetal protein is not clear at this time.

The results of our study show an association between expression of MT and other prognostic factors such as nuclear and histological grade and ER content. A close association of MT expression with cell proliferation and differentiation has been found in genital organs of the male rat suggesting a possible involvement of MT in supply or storage of zinc ions (Nishimura et al. 1990). It is possible that anaplastic and aggressive tumours with more cellular metabolic activity may have higher zinc and MT requirements.

MT overexpression has been recently implicated as an important factor in resistance to drugs by tumour cells (Andrews et al. 1987; Kelleey et al. 1988). Others, however, have found no significant difference in tumour MT content before and after cytotoxic chemotherapy, hence excluding MT as a major factor in resistance to drugs in ovarian cancer (Murphy et al. 1991).

In conclusion, our results suggest the use of MT as an additional biological marker in breast carcinoma is helpful in the characterization of more aggressive and less differentiated tumour groups.

References

Andrews PA, Murphy MP, Howell SB (1987) Metallothionein-mediated resistance in human ovarian carcinoma cells. Cancer Chemother Pharmacol 19:149

Benerjee D, Onasaka S, Cherian MG (1982) Immunohistochemical localization of metallothionein in cell nucleus and cytoplasm on rat liver and kidney. Toxicology 24:95–105

Bloom HJG, Richardson WW (1957) Histological grading and prognosis in breast cancer. A study of 1049 cases of which 359 have been followed for 15 years. Br J Cancer 9:359–377

Bremner I, Willians RB, Young BW (1977) Distribution of copper and zinc in the liver of the developing fetus. Br J Nutr 38:87–92

Cherian MG, Goyer RA (1978) Metallothioneins and their role in the metabolism and toxicity of metals. Life Sci 23:1–8

Eastman A, Richon VM (1985) Biochemical mechanisms of platinum antitumor drugs. Mc Brien DC, Slater TF (eds) Oxford IRL Press, pp 91

Kagi JRR, Nordberg M (1979) Metallothionein. Birkhause, Basel, pp. 48–116

Kagi JHR, Himmelhoch SR, Whanger PDD, Betheune JL, Vallee BL (1974) Equine hepatic and renal metalothioneins: purification, molecular weight, amino acid composition, and metal content. Biol Chem 249:3537–3542

Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481

Kelleey SL, Basu A, Teicher BA, Halker MP, Hamer DH, Lazo JS (1988) Overexpression on MT confers resistance to anticancer drugs. Science 241:1813–1815

Kontozoglou TE, Banerjee D, Cherian G (1987) Immunohistochemical localization of metallothionein in human testicular embryonal carcinoma cells. Virchows Arch [A] 415:545–549

Murphy D, Mcgown AT, Crowther D, Mander A, Fox BW (1991) Metallothionein levels in ovarian tumours before and after chemotherapy. Br J Cancer 63:711–714

Nartey N, Cherian MJ Banerjee D (1987) Immunohistochemical localization of metallothionein in human thyroid tumours. Am J Pathol 129:177–182

Nishimura H, Nishimura N, Tohyama C (1990) Localization of metallothionein in genital organs of the male rat. J Histochem Cytochem 38:927–933

Panemangalore M, Banerjee D, Onosaka S, Cherian MG (1983) Changes in intracellular accumulation and distribution of metallothionein in rat liver and kidney during postnatal development. Dev Biol 97:95–102

Peto P, Pike MC, Armitage P et al. (1977) Design and analyses of randomized clinical trials requiring prolonged observation of each patient. II. Analyses and examples. Br J Cancer, 35:1–39

Ryden L, Deutsh HF (1978) Preparation and properties of the major copper-binding component in human fetal liver. J Biol Chem 253:519–524

Schmid KW, Ellis IO, Gee JMV et al. (1993) Presence and possible significance of immunohistochemically demonstrable metallothionein over-expression in primary invasive ductal carcinoma of the breast. Virchows Arch [A] 422:153–159

Webb M, Cain K (1982) Functions of metallothionein. Biochem Pharmacol 31:137–142