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Prognostic Significance of the CaMBr1 Antigen on Breast Carcinoma: Relevance of the Type of Recognised Glycoconjugate

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An extensive study of the expression of the blood group-related antigen CaMBr1 has been performed by immunohistochemistry, immunoblotting and high performance thin layer chromatography both on frozen and paraffin-embedded (paraffin) samples from normal and neoplastic breast tissues. The glycolipid antigenic fraction (from frozen samples) was preferentially expressed on functioning breast epithelium. In a prospective series of 143 breast cancer cases CaMBr1 expression was associated, on frozen sections, with the transferrin receptor ($P = 0.01$), the positivity with oestrogen receptor immunochemical assay ($P = 0.06$), premenopausal status ($P = 0.06$) and node negativity ($P = 0.07$). Non-significant correlation with longer disease-free survival (DFS) was observed. In a retrospective series of 862 cases on paraffin sections the glycoproteic antigenic fraction was significantly associated with premenopausal status ($P < 0.05$) and lobular histotype ($P < 0.01$), but failed to predict survival, although a trend for longer DFS was observed for positive cases.
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INTRODUCTION

MBr1 IS AN IgM monoclonal antibody (MAb) produced in our laboratory in 1982 [1]. It was raised against a crude membrane preparation of the human breast cancer cell line MCF-7. The recognised epitope (CaMBr1) is present both on lipid and protein carriers and is expressed on a variety of non-neoplastic epithelial cells and epithelial tumours [2]; moreover, its expression is modulated on normal breast during the ovarian cycle and decreases in infiltrating breast carcinomas [3].

In breast cancer, MBr1 has been demonstrated to be a useful tool for diagnostic purposes, whether employed alone or in combination with other antibodies, in the differential diagnosis of effusions [4], or in the search for breast cancer cells in bone marrow biopsies [5–7]. By relying on the living cells' ability to internalise this antibody [10], therapeutic approaches have also been attempted by conjugating MBr1 with restrictocin [8] or the ricin A chain [9]. Furthermore, the prognostic usefulness of MBr1 has been proposed both in breast cancer [11, 12] and in small cell lung cancer [13].

In the first part of this paper we report a parallel analysis of the expression of glycolipid and glycoprotein antigenic fractions, as studied by immunoblotting, high performance thin layer chromatography (HPTLC) and immunohistochemistry, both on frozen and paraffin-embedded breast samples. In the second part, the prognostic relevance of MBr1 in breast cancer is studied by analysing the relationships with well-known prognostic factors and the survival outcome in two series of patients.

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MATERIALS AND METHODS

Materials

Production and characterisation of the murine MAb MBr1 have been described [1, 14]. Radiolabelling was performed using the ^{125}I-Bolton Hunter reagent (Amersham, Bucks, U.K.). The

anti-HER-2/*neu* protein polyclonal serum (diluted 1:500) was kindly provided by Dr Slamon (UCLA, Los Angeles, California, U.S.A.), whereas the anti-transferrin receptor (TRF-R) MAb and the oestrogen receptor immunochemical assay (ERICA) kit were purchased from Becton-Dickinson, (Mountain View, California, U.S.A.) and Abbott (Wiesbaden, Germany), respectively.

Immunochemical assay

The human breast cancer cell line MCF7 was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.).

Soluble extracts from normal and tumour specimens were obtained as described by Miotti *et al.* [14]. The extracts (approximately 300 μg of proteins) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and then immunoblotted, as described by Leoni *et al.* [15]. Glycolipid extracts from tumour specimens or the MCF7 cell line were obtained by tetrahydrofuran (THF) extraction [16]. The total glycolipid extracts were dried by evaporation under a nitrogen stream, then dissolved in methanol and applied to silica high performance thin layer chromatography (HPTLC) plates. After chromatography in chloroform/methanol/0.25% potassium chloride (5:4:1), the plates were soaked for 1 min in 0.1% of poly(isobutylmethacrylate) beads, dissolved in hexane and immunostained by ^{125}I-labelled MBr1 (10^6 cpm/ml) for 60 min at room temperature. After extensive washing with phosphate buffer the plates were submitted to autoradiography [15].

Patients

A prospective and a retrospective series of consecutive patients were studied. In the former, 143 consecutive patients, who were submitted to mastectomy or quadrantectomy plus radiotherapy at this institute in 1985, were analysed; median follow-up in this series was 53 months. The retrospective series, which has been described elsewhere [17], concerned 862 cases of operable breast cancer, submitted to mastectomy at this institute from January 1968 to December 1971; patients did not receive any adjuvant therapy and were homogeneously treated at relapse. The median follow-up was 19 years.

Oestrogen (ER) and progesterone (PgR) receptor levels were determined by the dextran-coated charcoal (DCC) method, as described previously [18]; the chosen cut-off valves for ER and PgR were 10 and 25 fmol/mg of protein, respectively.

Immunocytochemistry

The reactivity of MBr1 (ascitic fluid diluted 1:100 or purified antibody 10 μg/ml) was evaluated by immunoperoxidase (IPX) tests on histological sections of primary tumours in both series of patients, and by immunofluorescence (IF) on frozen sections in the prospective study only; both IPX and IF have been described elsewhere [1, 2]. The cases were defined positive if more than 10% of the tumour cells stained strongly.

Statistical analysis

The association between CaMBr1 expression and other variables was studied by contingency tables and evaluated by the χ^2 test.

Disease-free survival (DFS) time was considered as the time from the date of surgery to the date of the first unfavourable event (local recurrence, distant metastases, contralateral tumours) or the date of the last follow-up information. Survival time was considered as the time from the date of surgery to the date of death (for all causes). DFS and overall survival curves were traced by the product limit method [19] and compared by the log rank test. For the prospective study, the adjunctive contribution of CaMBr1 in predicting the prognosis when other prognostic variables were considered was evaluated by using a Cox regression model [20] and calculating the likelihood ratio.

RESULTS

Analysis of the molecules expressing the epitope recognised by MBr1

Firstly, by immunoblotting solubilised extracts from different primary breast carcinomas, we showed that MBr1 recognises an epitope shared by different molecules with a wide range of molecular weights. The diffusion of the labelling in the single bands suggested a high degree of glycosylation of these molecules. Moreover, a band migrating below the dye front, possibly corresponding to a glycolipid, was revealed in three out of five sampled tested (Fig. 1).

When material extracted from functioning (eight biopsies from pregnant or lactating women) or resting (8 cases) normal breast tissues was analysed by immunoblotting, the glycolipid antigen expression was found to be more frequent in the former than in the latter tissues (63 vs. 25%).

Two samples with different immunohistochemical behaviour, case no. 6 (positive on frozen but negative on paraffin sections) and no. 7 (positive on both) were tested by immunoblotting and HPTLC. Case no. 6 resulted positive by both procedures when the material was extracted from frozen samples; on the contrary, immunoblotting of case no. 7 showed immunoreactive molecules on both frozen and paraffin sections, and no glycolipid molecules were detected by HPTLC (Fig. 2, Table 1).

Prospective study

Out of 143 consecutive primary breast carcinomas, 109 (76%) and 66 (46%) expressed CaMBr1 on frozen and paraffin sections, respectively; 62 tumours (43%) were positive on both sections, 47 (33%) on frozen ones only, 4 (3%) on paraffin ones only and 30 (21%) tumours were negative on both. CaMBr1 expression on frozen sections was significantly associated with TRF-R

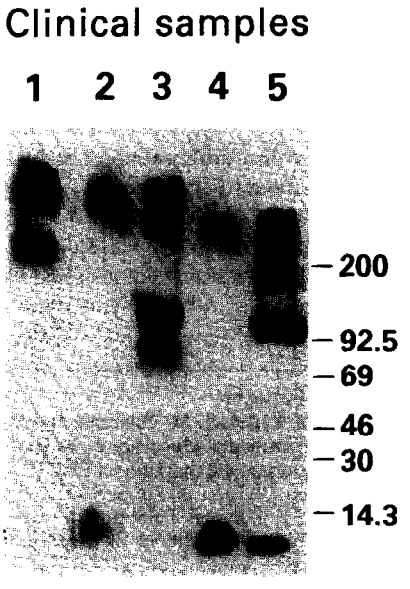

Fig. 1. Immunoblot of five breast carcinoma soluble extracts labelled with the MBr1 MAb (samples from 1 to 5).

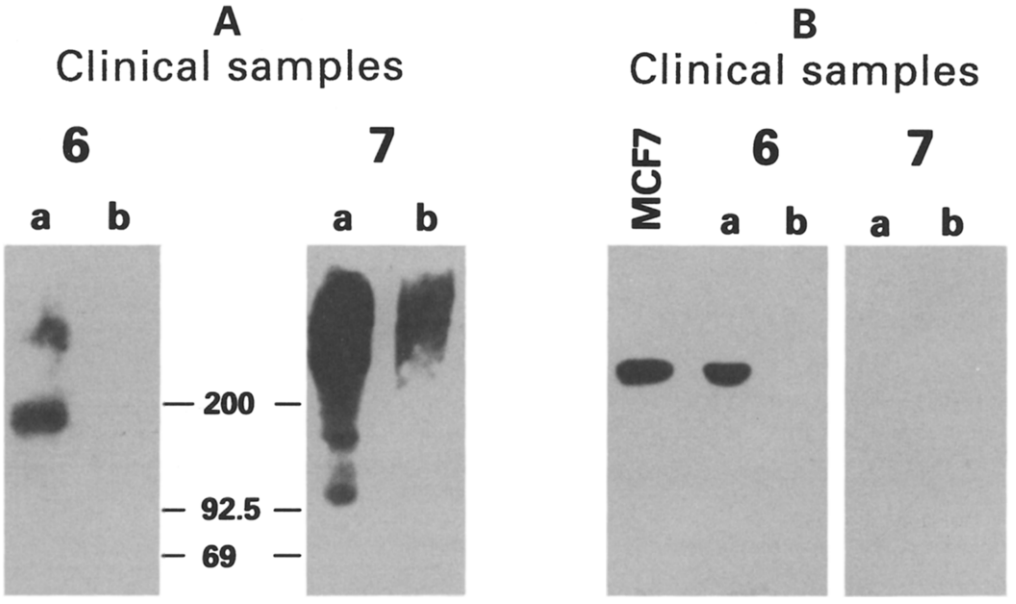

Fig. 2. A: Immunoblot of two breast carcinoma extracts solubilised from frozen sections (a) or paraffin sections (b). B: HPTLC of glycolipid extracts from the same two breast carcinomas obtained from frozen sections (a) or paraffin sections (b); (samples 6 and 7; MCF7 extract = positive control). Immunoreaction was performed with the MBr1 MAb.

($P = 0.01$) and approached significance with premenopausal status ($P = 0.06$), negative nodes ($P = 0.07$) and positive ERICA ($P = 0.06$), whereas its expression on paraffin samples approached significance only with a high tumour labelling index (TLI) ($P = 0.08$) and ER positivity ($P = 0.07$) (Table 2). DFS curves according to the MBr1 reactivity are shown in Fig. 3: patients with MBr1-positive tumours on paraffin sections tended to have a shorter DFS (a), whereas those with MBr1-positive tumours on frozen sections showed a better outcome (b); however, these differences were not statistically significant.

Multivariate analysis showed that ER, TRF-R and tumour size have an independent prognostic role as their hazard ratios significantly differ from 1.00, ER positivity, TRF-R positivity and T > 2.5 cm being the covariates associated with better outcome. The contribution of MBr1 reactivity on either frozen or paraffin sections was negligible; indeed, when MBr1 reactivity was entered into a model containing the abovementioned variables, the performance of the model did not increase significantly [likelihood ratio test = 0.02 ($P = 0.89$) and 0.04 ($P = 0.85$) for evaluation of frozen and paraffin sections, respectively].

Retrospective study

In the retrospective series, 862 consecutive paraffin sections of primary breast tumours were studied, 29% being MBr1-positive. A significant association was found between CaMBr1 expression and both menopausal status ($P < 0.05$) and lobular histotype ($P < 0.01$), whereas no relationship was evident with nodal status, tumour size, histological grade and *neu* expression (Table 3). All these variables, except histotype, had a significant impact on survival [17].

Univariate analysis showed that 19-year overall survival was

Table 1. Relationship between immunohistochemical and biochemical tests on frozen and paraffin samples: 2 cases

	Case no. 6		Case no. 7	
Test	Frozen	Paraffin	Frozen	Paraffin
Immunohistochemistry	+	−	+	+
Immunoblotting	+	−	+	+
HPTLC	+	−	−	−

Table 2. Prospective study: relationship between MBr1 reactivity and different prognostic variables

		% of MBr1-positive cases			
Variable	No. of cases	Frozen sections	*P*	Paraffin sections	*P*
Premenopausal	62	82	0.06	46	0.79
Postmenopausal	72	68		54	
Node negative	78	82	0.07	51	0.15
Node positive	64	69		39	
Tumour ≤ 2.5 cm	125	77	0.48	46	0.79
Tumour > 2.5 cm	16	69		50	
G1+G2	49	80	0.23	51	0.17
G3	31	68		35	
TLI ≤ 2.8	79	75	0.31	41	0.08
TLI > 2.8	19	63		63	
ER negative	32	69	0.26	34	0.07
ER positive	89	79		53	
ERICA negative	31	64	0.06	52	0.59
ERICA positive	104	81		46	
PgR negative	38	76	0.96	45	0.63
PgR positive	83	76		49	
EGF-R negative	62	71	0.20	50	0.64
EGF-R positive	44	82		46	
TRF-R negative	26	58	0.01	58	0.26
TRF-R positive	80	81		45	
Non-diploid	60	75	0.88	52	0.50
Diploid	38	74		45	

TLI, tumour labelling index; ER, oestrogen receptor; ERICA, oestrogen receptor immunochemical assay; PgR, progesterone receptor: EGF-R, epidermal growth factor receptor; TRF-R, transferrin receptor.

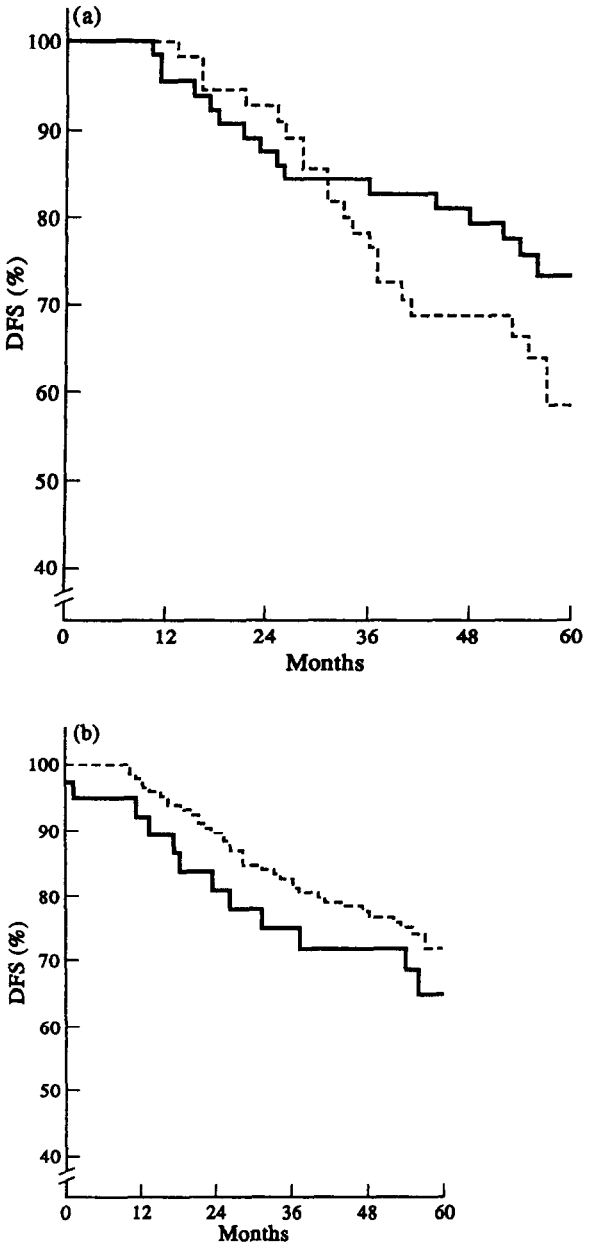

Fig. 3. Prospective series: DFS curves according to MBr1 reactivity evaluated on paraffin sections (a) or on frozen sections (b). ---- MBr1-positive cases; —— MBr1-negative cases.

not influenced by CaMBr1 expression (data not shown). To compare retrospective with prospective data, 5-year DFS curves were estimated: MBr1-positive cases exhibited a shorter 5-year DFS than MBr1-negative ones, although the difference is not statistically significant (Fig. 4).

DISCUSSION

MAb are useful tools both to characterise biological structures and to directly recognise antigenic structures, which may somehow be related to prognosis or other clinical parameters. However, the study of the expression of a MAb-recognised antigen involves a number of technical aspects which may influence the interpretation of results and lead to mistaken conclusions. The present report on the MAb-recognised antigen CaMBr1 is paradigmatic.

CaMBr1 is a glucidic blood group-related antigen carried by both lipids and proteins. When testing frozen tissues (by immunoblotting, HPTLC or immunohistochemistry), positivity is due to both glycolipid and glycoprotein fractions of the antigen; in contrast, positivity on paraffin sections is due to the glycoprotein fraction only, because of the extraction of the lipid material during fixing procedures.

Table 3. Retrospective study: relationship between MBr1-positive reactivity and different prognostic variables

Variable	No. of cases	% of MBr1-positive cases	*P*
Premenopausal	290	3	0.04
Postmenopausal	513	26	
Node negative	380	29	0.97
Node positive	458	29	
Tumour ≤ 2 cm	460	30	0.71
Tumour > 2 cm	248	28	
G1	81	26	0.31
G2	241	30	
G3	337	25	
Ductal type	732	27	0.003
Ductal-lobular	36	39	
Lobular type	88	43	
neu-negative	666	30	0.59
neu-positive	196	27	

Moreover, when testing paraffin sections, the rate of positive staining decreases as the age of the sample increases: 48% of positive cases were found in the prospective series (5 years old), 34% in a previously reported 10-year-old series [12] and 29% in the prospective series (20 years old). The reduction of reactivity might be due to denaturation of the antigen, despite fixation, or more intriguingly, to progressive changes of the disease phenotype itself.

The glycolipid antigenic fraction of CaMBr1 is associated with the functioning breast, during pregnancy or lactation [3]. In keeping with this observation is the appearance of CaMBr1 expression in proliferating bile ductules and cirrhotic liver cells, that can be regarded as a model of non-neoplastic growth stimulation [21].

TRF-R has been correlated previously with high histological

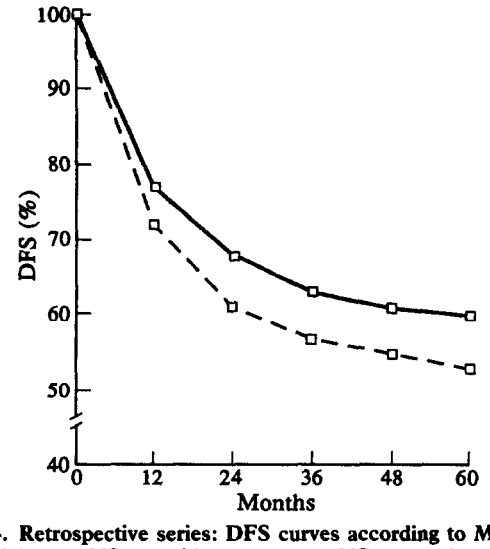

Fig. 4. Retrospective series: DFS curves according to MBr1 reactivity. ---- MBr1-positive cases; —— MBr1-negative cases.

grading [22] and the expression of the Ki67-recognised nuclear antigen [23], which are unfavourable prognostic factors. Surprisingly, in our series, TRF-R was significantly associated with a good prognosis. We suggest that, as a general rule, the expression of cellular membrane receptors (e.g. ER, PgR, TRF-R) may be interpreted as a marker of a differentiated cell population, the growth of which still respects positive or negative control systems; these tumours could, therefore, have a better prognosis. In the present paper, the significant or borderline association between CaMBr1 expression on frozen sections and TRF-R and ER, and the preliminary observation that MBr1-positive cases have a longer DFS (Fig. 3a), support this hypothesis.

When testing MBr1 on the paraffin sections (i.e. studying the glycoprotein antigenic fraction alone) no significant association was revealed with prognostic factors like nodal status, tumour size and grading both in prospective and retrospective studies. However, in the latter a significant relationship was shown between CaMBr1 and lobular histotype and premenopausal status. As the histological type had no prognostic relevance in this series [17], the meaning of this association is unclear; on the contrary, premenopausal status was associated with a worse 15-year survival rate (42.1 vs. 57.4% for postmenopausal [17]), thus suggesting a negative prognostic role of the glycoprotein CaMBr1 fraction. This is supported by the finding that, on paraffin sections in the prospective as well as in the retrospective study, CaMBr1 was associated with a shorter 5-year DFS. Although this association was very slight, not statistically significant and limited to the first follow-up period, it is consistent with data from a series of 40 hormone-treated metastatic breast cancer cases, where the presence of the CaMBr1 glycoprotein on the primary tumour was a marker of shorter survival [11] and, possibly, of resistance to hormonal manipulations [12].

Supporting the hypothesis that blood group-related determinants may represent useful prognostic markers, convincing evidence has been reported that the expression of H/Le^y/Le^b antigen in non-small cell lung cancer is related to a shorter survival [24], after adjustment for other prognostic factors. Moreover, Lee *et al.* [25] reported that in a series of non-small cell lung cancer patients with type A blood group, those with tumours that lost the ability to express the A-antigen had a significantly worse survival. Thus, it may be hypothesised that monoclonal antibodies, like MBr1, which recognise blood group-related antigens, resulting from altered protein glycosylation, may identify a general glycosylation pathway defect rather than a specific prognostic factor. Should this be the case, several cellular perturbations could be expected which would explain the correlation with the unfavourable prognosis.

In conclusion, where the expression of the blood group-related antigen CaMBr1 was evaluated on different tumour samples, we demonstrated that the contrasting results which were obtained could be attributed to technical issues. Thus, we suggest that better care is taken when testing the putative prognostic role of biological factors on retrospective series, since many variable factors may affect the analysis and possibly lead to misinterpretation of the data.

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