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MIB-1 Expression in breast carcinomas with medullary features

An immunohistological study including correlations with p53 and bcl-2

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Abstract Typical medullary carcinoma (TMC) is usually considered to have a more favourable prognosis than other types of infiltrating breast carcinomas. This is a biological paradox, since its clinical behaviour is not in agreement with its anaplastic morphology and high mitotic rate. It should be remembered that neoplastic growth reflects cell production minus cell loss, the latter being achieved by apoptosis. At present, *bcl-2* oncogene (apoptosis inhibitor) and *p53* gene are assumed to be involved in the regulation of cell death and tumour proliferation. Sixty breast carcinomas, initially indexed as medullary carcinomas, were re-classified using the diagnostic criteria given by Ridolfi. This review yielded 13 typical (TMC), 24 atypical (AMC), and 23 non-medullary carcinomas (NMC). Following antigen retrieval by microwave treatment, immunohistochemical analyses, using MIB-1, p53 and bcl-2 monoclonal antibodies were performed on serial sections from formalin-fixed, paraffin-embedded specimens. TMC revealed the highest incidence of intense p53 positivity, and the highest mean MIB-1 index, and absence of the apoptosis-inhibitor protein bcl-2. These results suggest the presence of a higher overall cell turnover in TMC than in AMC and NMC. Increased apoptosis balancing the increased cell proliferation might be among the possible explanations for the more favourable prognosis in TMC.

Key words bcl-2 · MIB-1 · p53 · Proliferative activity · Typical medullary carcinoma

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Introduction

Medullary carcinoma of the breast is a relatively uncommon type of infiltrating ductal carcinoma. The features of typical medullary carcinoma (TMC) are well-described by Ridolfi et al. [40]. Several studies have reported that TMC denote a relatively favourable prognosis compared with other subtypes of invasive breast carcinoma [39, 40, 43]. However, conventional histological prognostic parameters, such as nuclear grade and mitotic rate, indicate that medullary carcinoma is a high grade neoplasm.

This motivated us to search for biological characteristics that might explain the discrepancy between histological appearance and favourable outcome in this particular histological type.

Immunohistochemical detection of proliferative activity has shown to be of prognostic value in a variety of malignancies [14, 34, 42], including breast cancer [4, 25]. Introduced by Gerdes et al. [12, 13], anti-Ki-67 is a monoclonal antibody that reacts with a nuclear antigen expressed in all active phases of the cell cycle (G_1, S, G_2 and M) but absent in quiescent cells (G_0). Development of the Ki-67-equivalent monoclonal antibody MIB-1, described by Cattoretti et al. [5], allows retrospective studies based on the immunohistochemical staining of routinely processed, paraffin-embedded tissue.

The maintenance of neoplastic growth reflects a balance between cell proliferation and cell death, the latter sometimes conspicuous. Apoptosis, in which the cell and nucleus condense and fragment, is believed to be the most important mechamism of cell death [28], and its inhibition may increase cell survival and facilitate the development of the neoplasm. At present, the *bcl-2* oncogene and the *p53* suppressor gene are assumed to be involved in the regulation of cell death and tumour proliferation: *bcl-2* is believed to be important in suppressing apoptosis [20], whereas wild-type *p53* is considered to induce apoptosis under certain conditions [33, 46]. Although both of these genes have been associated with the process of apoptosis, their relationship in the process is largely unknown.

It has been shown that wild-type p53 protein acts as a regulatory checkpoint in the cell cycle, arresting cells in the G_1 phase [8, 30, 32]. *p53* Gene alterations are associated with the removal of the p53-mediated negative growth signal and may facilitate the uncontrolled proliferation of neoplastic cells. When considering immunohistochemical studies of p53 in tumours it is important to remember that different mutations in the *p53* gene vary in phenotype. The majority of *p53* gene mutations are missense mutations [35] causing an abnormal accumulation of p53 protein within the nuclei, allowing it to be detected using immunohistochemistry [30]. Conversely, as a result of frameshift or nonsense mutations, the p53 protein is often missing or unstable and thus escapes immunohistochemical detection [2, 19]. Most of the available antibodies to p53 protein (including DO7 antibody) recognize both the wild-type and the mutated protein, not being specific for the latter. Thus, the detection of a p53 positive phenotype, frequently although not absolutely, reflects the occurrence of a gene mutation. This discrepancy could be due to either an upregulated expression of the wild-type gene or to stabilization of p53 by mechanisms other than mutation through interactions with viral or cellular proteins, such as MDM2 [27, 36]. Consequently, immunohistochemical detection of p53 accumulation cannot be taken as definite evidence of gene mutation, and conversely, *p53* gene mutations may occur without p53 accumulation.

To address the apparent contradiction of histological features and clinical outcome, the aim of the present study was to evaluate whether TMC might have immunohistochemical characteristics associated with the regulation of neoplastic growth.

Materials and methods

The histopathological material was identical to that used in a previous study of breast carcinomas with medullary features [23]. Briefly, it comprised 60 breast carcinomas, previously diagnosed as medullary carcinoma for the period from 1971 through 1995.

Ten serial sections were produced from each formalin-fixed, paraffin-embedded tumour sample, and were stained for immunoreactivty to MIB-1 (Immunotec, Marseille, France), p53 (DO7, Dako, Glostrup, Denmark), and bcl-2 (Dako) antibodies using the standard avidin–biotin (ABC) method. LCA (leucocyte common antigen) antibody, which recognizes lymphoid cells, was used in each case as a control for tissue immunoreactivity. Simultaneous staining of the entire series was performed for each antibody, and negative controls included substitution of monoclonal primary antibody with mouse myeloma proteins of the same subclass and concentration as the monoclonal antibody.

Sections were deparaffinized in xylene, rehydrated through graded alcohols and incubated for 2×5 min in citrate buffer (pH = 6.0) in a household microwave oven at 800 W. After microwave exposure the slides were allowed to cool down to room temperature. The slides were briefly washed with TRIS buffered saline (TBS, pH = 7.4) and incubated for 20 min with 3% hydrogen peroxide in water to block endogeneous peroxidase activity. The p53 and bcl-2 antibodies were used at 1:200 dilutions, and MIB-1 antibody at 1:100, for 30 min incubation at room temperature. Biotinylated anti-mouse/rabbit antibody (Dako) at a dilution of 1:100 was used as linking molecule. Finally, after washing, avidin–biotin complex (Dako) was applied and aminoethyl carbazole was used for visualization. The sections stained for MIB-1 were evaluated in a systematical, random sequence at high magnification (× 780, 60× oil immersion lens). The images were projected to the table using a standard microscope with projection attachment.

A median of 15 fields of vision (range 10–32) were sampled systematically by moving with a fixed distance between fields. An average number of 297 cells (range 117–721) were counted, using a counting frame and an unbiased counting rule [16]. Areas of necrosis were avoided.

Tumour cells were considered to be positive for MIB-1 if there was any staining of the nucleoplasm or nucleoli, regardless of staining intensity. The MIB-1 index was calculated as the fraction of positive tumour cells.

The cases were considered to be p53 positive if over 10% of the tumour cells manifested a definite nuclear staining without any attempt to quantify the staining intensity. Bcl-2 reactivity was scored positive if there was moderate or strong cytoplasmic staining in the majority of the cells.

Comparisons of grouped means were carried out using Student's *t*-test, and analyses of contingency tables was performed using the Chi-square test or Fisher's exact test. The relationship between continuous variables was evaluated by standard linear regression analysis. The level of significance for all statistical tests was chosen as $2P<0.05$.

Results

Tumour specimens were reclassified by M.L.J. and H.K. using the six strictly defined histopathological features given by Ridolfi et al. [40]. This review yielded 13 TMC, 24 atypical carcinomas (AMC), and 23 non-medullary carcinomas (NMC). The clinicopathological information listed in Table 1 was obtained from the histopathological reports and the Danish Breast Cancer Cooperative Group (DBCG) registry.

The most important prognostic factors, such as tumour size and axillary lymph node status, were not significantly different among the three groups. Thus, neither

Table 1 Clinicopathological characteristics of patients with breast carcinoma with medullary features (*TMC* Typical medullary carcinoma, *AMC* Atypical medullary carcinoma, *NMC* Non medullary carcinoma)

	TMC	AMC	NMC
Age (mean, years)	51	64	61
Range	36–66	32–92	34–92
Menopausal status			
Premenopausal	5	5	7
Postmenopausal	8	19	16
Primary surgery			
Mastectomy	9	22	20
Lumpectomy	4	2	3
Lymphnode status[a]			
Positive	4	8	6
Negative	6	9	7
Unknown	3	7	10
Tumour size (median, mm)	29	25	25
Range	10–40	14–100	10–50
Oestrogen receptor status[b]			
Positive	0	2	5
Negative	13	22	18

[a] ≥6 lymph nodes removed

[b] Immunohistochemical analyses; positive status: nuclear staining in >10% of tumor cells

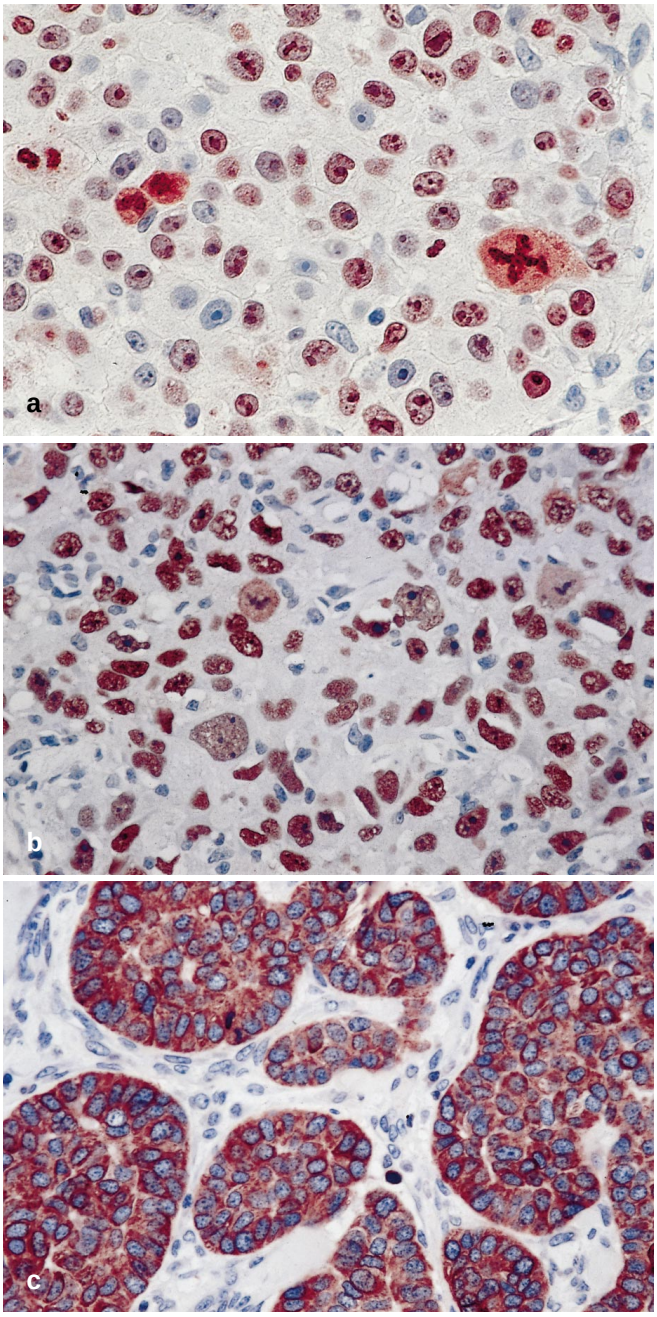

Fig. 1a MIB-1 Expression in a typical medullary carcinoma; **b** p53 accumulation in a typical medullary carcinoma; **c** *bcl-2* expression in a non-medullary carcinoma

tumour size nor axillary lymph node status correlated with the consensus diagnosis of TMC, AMC or NMC. The mean age for TMC (51 years) was significantly lower than the mean ages for AMC (64 years) and NMC (61 years), respectively ($2P = 0.002$). In spite of the relatively small study population and inhomogeneity of the treatment regimens our previous study [23] clearly showed a survival advantage of TMC compared with AMC and NMC. It was also appearent from this study that the majority of patients who died of their disease did so within 5 years of diagnosis in both AMC and NMC patients. The median follow-up time for TMC was 60 months (range 3–253 months). Recurrence was observed in 1 patient with TMC, who presented with an invasive ductal carcinoma in the same breast at the time of initial diagnosis. In AMC and NMC recurrence was observed in 16 cases and 8 cases, respectively.

Positive staining for MIB-1 was evident in each section as a diffuse nuclear staining, with accentuation of the nucleoli and on chromosomes in mitotic cells (Fig. 1a). The mean MIB-1 index of TMC (61% ± 10% [mean, ± standard deviation (SD)] was significantly higher than those of AMC (40%, ± 17%) and NMC (40%, ± 22%) ($2P = 0.004$).

Of the 60 tumours studied, 36 (60%) gave a positive nuclear reaction for p53 in more than 10% of the tumour cells. Heterogeneity was common with regard to both the staining intensity and the number and distribution of stained cells. Occasionally, a few scattered epithelial cells in the surroundings were stained. However, the staining intensity was obviously weaker than in the neoplastic tissue. Although a cut-off point of 10% was chosen a striking feature was intensive nuclear p53 staining in over 75% of the tumour cells (Fig. 1b), which was observed in 69% of TMC, 39% of AMC, and 13% of NMC ($2P = 0.003$).

The bcl-2 reactivity was observed in lymphoid infiltrates in all tumours. This “noise” reactivity could be excluded from the section if bcl-2 reactivity was compared with that of LCA. Staining for LCA was recognized in each section in lymphoid cells, whereas no reactivity was observed in non-haematopoietic tissue. The bcl-2 immunostaining was always localized in the cytoplasm of tumour cells, but a wide inter-tumour variation was observed. The bcl-2 staining of tumour tissue was scored as negative if only weak staining or none at all was present. Moderate to strong staining of the cytoplasm in almost all cells (Fig. 1c) was detected in 14 (23%) of the tumours. Lobular and ductal epithelial cells in the surroundings showed intense bcl-2 reactivity regardless of the bcl-2 reactivity in the neoplastic tissue. TMC were all bcl-2 negative. In contast, 25% of AMC and 36% of NMC showed moderate to strong cytoplasmic staining in the majority of tumour cells ($2P = 0.03$). The results of the immunohistochemical analyses are shown in Fig. 2a–c.

In the entire series, the mean MIB-1 index in p53-positive tumours was significantly different from the mean MIB-1 index in p53-negative tumours (54% versus 40%; $2P = 0.002$). An inverse relationship was observed between MIB-1 index and age ($r = –0.21$, $2P<0.001$), and the mean MIB-1 index of oestrogen receptor (ER)-positive tumours (19%) was significant lower than that of ER-negative tumours (48%; $2P<0.001$). A significant inverse correlation between p53 status and bcl-2 status was observed ($2P = 0.04$). No significant difference in mean MIB-1 index according to bcl-2 status was demonstrated, whereas bcl-2 reactivity was positively correlated to ER positivity ($2P<0.001$). We found no significant relationship between p53 or bcl-2 status and the clinico-

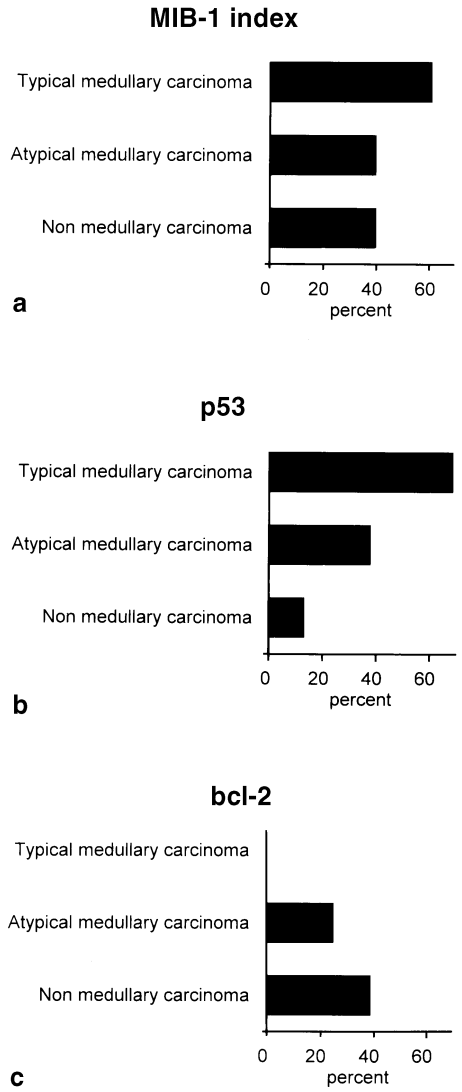

Fig. 2a Mean MIB-1 index according to Ridolfi classification. TMC: μ = 61%, 95% C.I. 55–67%; AMC: μ = 40%, 95% C.I. 33–48%; NMC: μ = 40%, 95% C.I. 31–50%. **b** Incidence of intensive nuclear p53 staining in >75% of the tumour cells according to Ridolfi classification. c Incidence of moderate to strong cytoplasmic bcl-2 staining in >75% of the tumour cells according to Ridolfi classification

pathological variables such as age, tumour size and lymph node status.

Discussion

The suitability of MIB-1 antibody for the detection of proliferating cells has previously been reported [29, 44]. Furthermore, in previous reports we have shown that when microscopic evaluation of systematic, randomly selected fields of vision is carried out the MIB-1 index is a reliable and reproducible estimate of proliferative activity [24]. The relatively high mean MIB-1 index of the subtypes investigated are in accordance with the presence of numerous mitotic figures and an apparent rapid growth fraction for medullary carcinomas compared with other types of invasive breast cancer [6]. An interesting observation in the entire series was the inverse correlation between patient age and MIB-1 index, a prominent feature particularly in TMC, characterized by a significantly lower mean age and a significantly higher mean MIB-1 index than in AMC and NMC.

Several investigators have studied the prognostic value of immunohistochemical p53 accumulation in breast carcinomas [3, 11, 21, 41]. However, the results of these studies are divergent, and it is difficult to compare the results obtained by different studies because of methodological and technical differences. The major problem with immunohistochemical studies of p53 arises in the interpretation, as the methodological approaches in the immunohistochemical techniques are very variable (different antibodies, use of different methods to retrieve antigenicity and different fixatives). Furthermore, methods for quantification of p53 reactivity and choice of cut-off values (more commonly staining of at least 10% of the tumour cells) to define p53 accumulation may affect the final results significantly.

Our results show that nuclear p53 accumulation is a common phenomenon in medullary carcinoma, which is in accordance with the very few previous studies reporting on this specific histological type [7, 9, 21, 31]. Furthermore, the results of our study show striking differences in nuclear p53 accumulation among TMC and AMC/NMC. Thus, TMC – which has the most favourable prognosis – has the highest incidence of p53 accumulation. We cannot determine whether these p53-positive cases were accompanied by *p53* gene mutation, because we used monoclonal DO7 antibody, which recognizes an epitope shared by both wild-type and mutant p53 protein. The results suggest, however, that TMC is either more predisposed to *p53* gene mutation or to stabilizing wild-type p53 protein.

The low incidence of bcl-2 positivity in AMC/NMC is in accordance with previous reports showing relative absence of bcl-2 in poorly differentiated breast cancers [22, 26].

The inverse correlation between p53 and bcl-2 is surprising but has also been found in other studies [15, 17, 18, 22, 38]. Hurlimann et al. [21, 22] demonstrated that medullary carcinomas are almost always positive for p53 protein but rarely positive for bcl-2, findings confirmed in our study where 9 of 13 bcl-2-negative TMCs showed intensive nuclear p53 staining in more than 75% of the tumour cells. Although bcl-2 protein was the first molecule known to be involved in the regulation of cell death [20] it is now clear that wild-type p53 protein also induces apoptosis [46]. Previous data suggest that high levels of wild-type p53 can decrease bcl-2 expression, which may have important implications for the mechanism of p53-induced apoptosis [33]. Haldar et al. [17] reported that analysis of multiple human breast cancer cell lines with antibodies against p53 and bcl-2 revealed that expression of the two proteins is in most cases reversed. Because the immunohistochemically detectable p53 indi-

cates a mutated protein, they suggest that mutant p53 may maintain its down-regulating effect on bcl-2 expression. Regardless of whether the high incidence of p53 positivity in TMC represents accumulation of wild-type or mutant p53, the effect could be enhancement of apoptotic cell death, which potentially might counteract the high proliferative activity seen in TMC.

In 1977, Ridolfi et al. [40] proposed specific criteria for the diagnosis of TMC and reported that the 10-year survival for patients with TMC was better than for patients with AMC or NMC. The prognostic significance of these criteria have been confirmed in another study [23] when applied to the current series. The clinicopathological and immunohistochemical characteristics investigated here are very similar for AMC and NMC. Indeed, there were no statistically significant differences between AMC and NMC in MIB-1 index and incidence of p53 accumulation and bcl-2 reactivity. These findings supports the suggestions that the designation AMC be discarded [39, 43], and these tumours regarded instead as poorly differentiated ductal carcinomas (NMC), since the survival figures are comparable to those for this tumour type. Other reports have shown an increased proliferative activity in p53-positive tumours [1, 3, 10] analogous to the results of our study, and several studies have demonstrated that detection of p53 accumulation [11, 41] and a high proliferation index [4, 25] strongly correlate with decreased survival in breast cancer. However, our results indicate that these immunohistochemical characteristics are not associated with biological agressiveness in TMC, and careful consideration is warranted before this specific tumour type is included in studies evaluating newer prognostic variables in breast cancer. Evaluation of the prognostic impact of the immunohistochemical analyses in the specific subtypes was not possible in the present study, however, owing to the rather small number of tumours in each group.

The antibodies used in this study did not reveal a specific phenotype for TMC. However, in differential diagnostic situations we suggest immunohistochemical staining as a diagnostic tool. When a medullary cancer is suspected, negative ER and bcl-2 staining, p53 reactivity in the majority of tumour cells, and an extremely high proliferative activity detected by MIB-1 antibody makes the diagnosis of typical medullary carcinoma more likely. Despite strict application of the Ridolfi criteria, including the lack of infiltration of tumour margins, it is striking that the extreme high proliferative activity observed apparently does not affect the clinical outcome in this specific tumour type. The underlying causes for the clinical differences between TMC and AMC/NMC are still unknown, but among the possible explanations for the discrepancy is considerable cell loss through apoptosis, indicated by the totally absence of bcl-2 reactvity in TMC. Increased apoptotic rate might influence tumour progression, since it may have an effect not only in the primary tumour but also on the ability of tumour cells to survive in the circulation and in metastatic sites. This study did not examine whether absence of the apoptosis-inhibitor protein bcl-2 in the tumours is associated with an increased number of apoptotic cells. Until recently, apoptosis within tissues has been measured histologically by morphological assessment. The development of in situ end-labelling (ISEL) techniques [45] has greatly enhanced and facilitated the detection and quantitation of apoptosis in tissue sections. By this method the 3'-OH ends of the small DNA-fragments produced during apoptosis act as sites for incorporation of biotinylated deoxynucleotides. Furthermore, Panchalingan et al. [37] have demonstrated that an antibody directed against a cytosolic apoptosis-specific protein (ASP) expressed exclusively within apoptotic cells is useful for the detection of apoptotic cells in formalin-fixed, paraffin-embedded tissue. Additional studies, using the above mentioned methods, are needed to clarify whether absence of bcl-2 reactivity is associated with increased number of apoptotic cells.

Future studies should be directed at showing whether different mechanisms are responsible for the immunohistochemical detection of p53 accumulation in TMC and AMC/NMC, for further elucidation of differences between these lesions. The possibility of p53 accumulation in TMC in the absence of gene mutation must be considered. Careful molecular analysis of the *p53* gene in the present series of carcinomas with medullary features is now in progress, in an attempt to establish the basis of the observed p53 accumulation.

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