The expression pattern of MUC1 (EMA) is related to tumour characteristics and clinical outcome of invasive ductal breast carcinoma

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Aims: To clarify MUC1 patterns in invasive ductal breast carcinoma and to relate them to clinicopathological parameters, coexpression of other biological markers and prognosis.

Methods and results: Samples from 243 consecutive patients with primary ductal carcinoma were incorporated into tissue microarrays (TMAs). Slides were stained for MUC1, oestrogen receptor (ER), progesterone receptor (PR), Her2/neu, p53 and cyclin D1. Apical membrane MUC1 expression was associated with smaller tumours ($P = 0.001$), lower tumour grades ($P < 0.001$), PR positivity ($P = 0.003$) and increased overall survival (OS; $P = 0.030$). Diffuse cytoplasmic MUC1 expression was associated with cyclin D1 positivity ($P = 0.009$) and increased relapse-free survival (RFS; $P = 0.034$). Negativity for MUC1 was associated with ER negativity ($P = 0.004$), PR negativity ($P = 0.001$) and cyclin D1 negativity ($P = 0.009$). In stepwise multivariate analysis MUC1 negativity was an independent predictor of both RFS [hazard ratio (HR) 3.5, 95% confidence interval (CI) 1.5, 8.5; $P = 0.005$] and OS (HR 14.7, 95% CI 4.9, 44.1; $P < 0.001$).

Conclusions: The expression pattern of MUC1 in invasive ductal breast carcinoma is related to tumour characteristics and clinical outcome. In addition, negative MUC1 expression is an independent risk factor for poor RFS and OS, besides 'classical' prognostic indicators.

Introduction

MUC1 (episialin, epithelial membrane antigen, CA15-3 antigen) is a highly O-glycosylated mucin-like trans- membrane glycoprotein encoded by a gene on chromosome 1q21.[1] This protein has a very large extracellular domain consisting mainly of 20 amino acid tandem repeats, a transmembrane domain and a cytoplasmic tail.[2–4]

In most normal glandular epithelial cells, MUC1 is expressed on the apical surface.[5] *In vitro* and *in vivo* studies have described cell adhesion inhibition as well as increased metastatic and invasive potential of tumour cells associated with overexpression of MUC1.[6–8] In

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MUC1-deficient mice primary breast tumours have a significantly lower growth rate.[9] Overexpression of an underglycosylated form of MUC1 occurs in nearly all breast carcinomas.[10–12]

Using numerous different antibodies and scoring methods, many authors have described correlations between MUC1 expression and oestrogen receptor (ER) status, grade of differentiation and prognosis.[13–15] In contrast with the *in vitro* work, most of these studies have shown a better outcome for patients overexpressing MUC1. Four studies, however, found no relation between MUC1 expression and outcome.[16–19] These differences may be explained by the complex scoring system used, the different affinity of the applied antibodies for the glycosylated isoforms of MUC1 and the wide range of histopathological phenotypes of breast carcinoma with different clinical and prognostic implications.[20]

Therefore, we used a monoclonal antibody directed at the protein backbone of MUC1 (mAb 214D4), which is relatively insensitive to the degree and make-up of glycosylation of the molecule[21] to study five patterns of MUC1 expression in primary ductal carcinomas which were predefined by two of the authors (C.P and J.L.P). To test the potential of this scoring method, it was applied to a set of primary invasive ductal breast carcinomas (not otherwise specified) arranged in a tissue microarray (TMA) and the MUC1 expression patterns were related to clinicopathological parameters, a series of well established biological markers and prognosis. This scoring method has also been applied to a set of ductal carcinomas *in situ* (DCIS).[22]

Materials and methods

PATIENTS

Consecutive patients (n = 243) treated for a primary operable invasive ductal carcinoma of the breast (not otherwise specified) at the University Medical Centre Groningen between January 1996 and December 2001 were included in this study. Patient and tumour characteristics and data on follow-up were obtained retrospectively from hospital records and are summarized in Table 1. The median follow-up was 60.5 months (range 0.4–108.2). Follow-up was performed according to the regional follow-up guidelines (http://www.ikcnet.nl/page.php?id=97). During follow-up 12 patients developed a local recurrence after a median follow-up of 26.7 months. Thirty-three patients developed distant metastasis after a median follow-up of 36.7 months. In total, 41 patients presented with a relapse with a median relapse-free survival

Table 1. Patient and tumour characteristics

	n	%
Age at diagnosis, median (range)	58 (27–89)	
Menopausal status		
Premenopausal	75	30.9
Postmenopausal	168	69.1
Family history		
Positive	34	14.0
Negative	157	64.6
Unknown	52	21.4
Therapy		
Breast-conserving therapy	145	59.6
Mastectomy	98	44.9
Axillary nodal status		
Negative	131	53.9
Positive	107	44.0
Not assessed	5	2.1
Pathological tumour size (mm), median (range)	20 (2–140)	
Pathological tumour stage		
T1	109	44.9
T2	109	44.9
T3	18	7.4
Unknown	7	2.9
Grade of differentiation		
I	57	23.5
II	110	45.3
III	75	30.9
Missing	1	0.4
Adjuvant chemotherapy		
Yes	61	25.1
No	182	74.9
Adjuvant hormonal therapy		
Yes	87	35.8
No	156	64.2

n, Number of cases; T1, tumour diameter <20 mm; T2, tumour diameter ≥20 mm but <50 mm; T3, tumour diameter ≥ 50 mm.

(RFS) of 27.3 months; 20 patients died due to breast cancer with a median overall survival (OS) of 34.1 months.

TISSUE MICROARRAY CONSTRUCTION

From a paraffin block of each tumour, three 0.6-mm core samples of the most representative tumour area were included in a TMA. The technique of TMA production has been described and validated for breast carcinoma by others.[23,24] In brief, the most representative tumour area was marked on the original haematoxylin and eosin (H&E)-stained section. Using this section for orientation, three 0.6-mm core punches were taken from the selected area in the donor blocks and mounted in a recipient block, using a manual TMA device (Beecher Instruments, Silver Springs, MD, USA).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry for MUC1, ER, progesterone receptor (PR), Her2/neu, cyclin D1 and p53 was performed on these sections. The antibodies and antigen retrieval methods used are summarized in Table 2. The immunohistochemical protocol was as follows: sections were deparaffinized in pure xylene,

rehydrated in decreasing concentrations of ethanol and washed in distilled water. Antigen retrieval was performed. Endogenous peroxidase was blocked by incubating in 3% perhydrol for 30 min. The primary antibody diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) was incubated for 1 h, after which the secondary (1 : 100 diluted in PBS containing 1% BSA and 1% AB-serum) and tertiary (1 : 100 diluted in PBS containing 1% BSA and 1% AB-serum) antibodies were incubated for 30 min each. Visualization was performed using the diaminobenzidine tetrahydrochloride/peroxidase reaction. Counterstaining was performed using haematoxylin. Sections were dehydrated using increasing concentrations of alcohol and were mounted.

EVALUATION OF IMMUNOHISTOCHEMISTRY

Scoring of immunohistochemistry was performed by a resident (B.v.d.V.) and randomly verified by an experienced pathologist (J.W.). ER, PR, p53 and cyclin D1 were graded based on the percentage of tumour cells showing nuclear immunopositivity. ER,

Table 2. Antibodies and antigen retrieval methods

Antibody	Clone	Supplier	Dilution	Antigen retrieval	Secondary antibody	Supplier	Tertiary antibody	Supplier
MUC1	214D4	Dr J. Hilkens*	1 : 100	–	RAMPO	Dako	GARPO	Dako
ER	6F11	Ventana	†	Tris–HCl 0.1 M (pH 9.5) 30 min 98 °C microwave	RAMBIO	Dako	SARBIO	Dako
PR	1A6	Ventana	†	Tris–HCl 0.1 M (pH 9.5) 30 min 98 °C microwave	RAMBIO	Dako	SARBIO	Dako
p53	BP-53-12-1	Biogenix	1 : 800	Tris–HCl 0.1 M (pH 9.5) 30 min 98 °C microwave	RAMBIO	Dako	SARBIO	Dako
Cyclin D1	SP4	Neomarkers	1 : 50	Tris–HCl 0.1 M (pH 9.5) 30 min 98 °C microwave	RAMBIO	Dako	SARBIO	Dako
Her2/neu	CB11	Ventana	†	Tris–HCl 0.1 M (pH 9.5) 30 min 98 °C microwave	RAMBIO	Dako	SARBIO	Dako

ER, Oestrogen receptor; PR, progesterone receptor; RAMPO, rabbit antimouse polyclonal; RAMBIO, rabbit antimouse biotin; GARPO, goat antirabbit polyclonal; SARBIO, swine antirabbit biotin; –, no antigen retrieval neccesary.
*Gift from Dr J. Hilkens, Division of Tumour Biology, the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 10066 CX Amsterdam, the Netherlands.
†Prediluted by supplier.

PR and cyclin D1 were considered positive if nuclear staining was present in $\geq 10\%$ of the cells and p53 was considered positive when there were $> 30\%$ of positively stained nuclei. Her2/neu expression was graded as recommended by the manufacturer's scoring guidelines: 0, no staining at all or membrane staining in $< 10\%$ of tumour cells; 1+, faint/barely perceptible partial membrane staining in $> 10\%$ of tumour cells; 2+, weak to moderate complete membrane staining in $> 10\%$ of tumour cells; 3+, intense complete membrane staining in $> 10\%$. Her2/neu was considered positive if the score was 3+. MUC1 was graded according to the five expression patterns depicted in Figure 1. MUC1 expression was
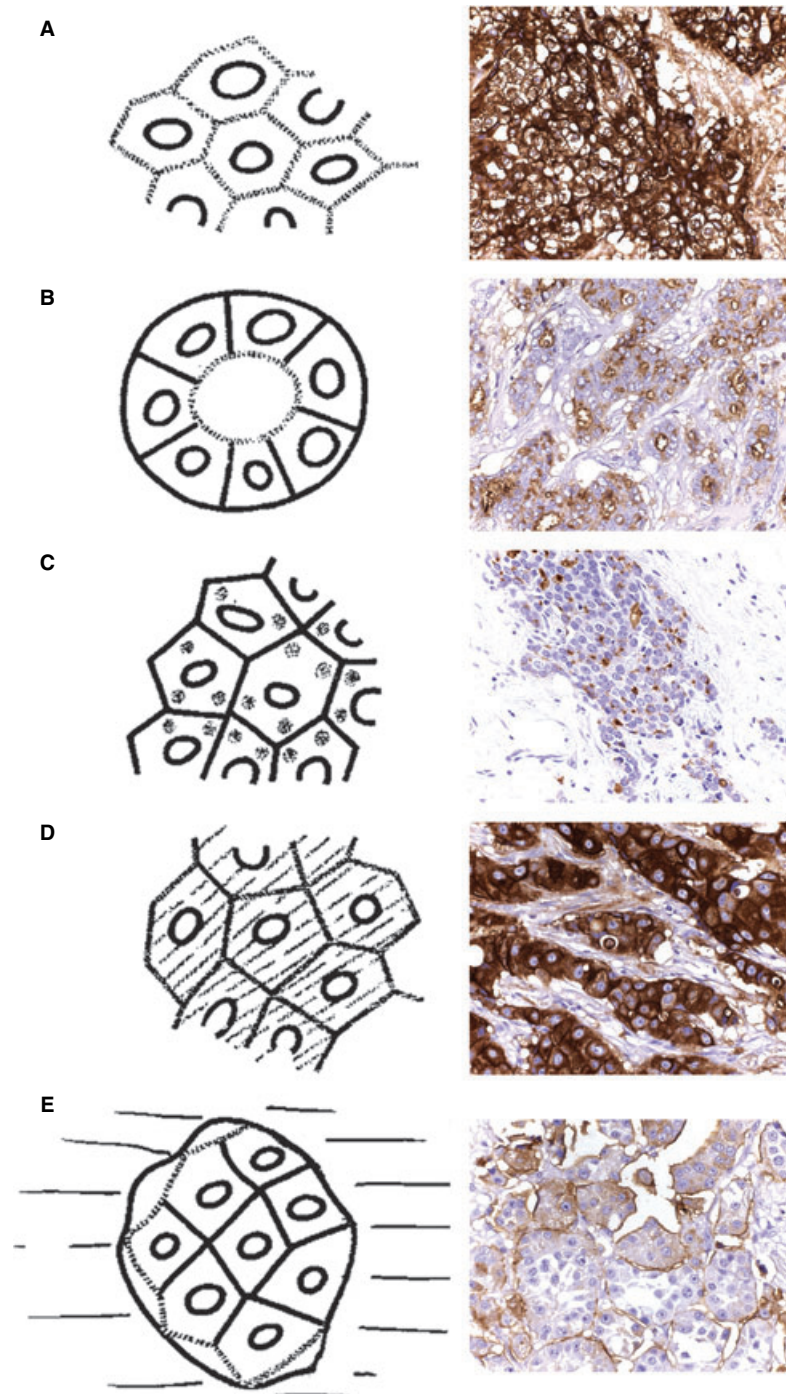

Figure 1. MUC1 immunoreactivity patterns as classified in this study. **A,** Entire membrane. **B,** Apical. **C,** Focal cytoplasmic. **D,** Diffuse cytoplasmic. **E,** 'Inside-out'.

considered positive if there was staining in >10% of tumour cells.

DATA ANALYSIS

Data analysis was performed using the SPSS 12.0.1 statistical package (SPSS Inc., Chicago, IL, USA). χ^2 tests were used to evaluate the association of MUC1 expression with clinicopathological parameters and biological markers. Because this was an exploratory analysis, a strict significance level was used. Only *P*-values (uncorrected) <0.01 were included. If applicable, Fisher's exact test was used. In a second analysis, Kaplan–Meier curves were plotted and log-rank scores were calculated. In this analysis, $P < 0.05$ was considered to be significant. After this, the six expression patterns were simplified into three sub-groups according to expression location: entire membrane, apical membrane and 'inside-out' expression were classified as membrane expression; diffuse cytoplasmic and focal cytoplasmic were classified as cytoplasmic expression; tumours negative for MUC1 were classified as negative. These groups and other well-established prognostic indicators were entered into univariate Cox regression analysis to analyse the relationship with RFS and OS. Variables from the univariate analysis with a *P*-value of <0.05 were then entered in a stepwise multivariate Cox regression analysis to investigate the relationship with RFS and OS.

Results

TISSUE MICROARRAY AND IMMUNOHISTOCHEMISTRY

Of the 243 cases included, the tissue cores of 237 cases were adequately represented in the TMA. Immuno-histochemistry could be evaluated in all cases (100%, $n = 237$) for MUC1, p53 and cyclin D1, in 235 cases (99.2%) for Her2/neu, in 232 cases (97.9%) for ER and in 230 cases (97.0%) for PR.

In the assessable cases, MUC1 was expressed in 221 cases (93.2%) showing either a single or a combination of expression patterns. Sixteen cases (6.8%) did not show any expression of MUC1. Entire membrane expression was seen in 48 cases (20.3%). Sixty-four cases (27.0%) showed apical expression. In 21 cases (8.9%), focal cytoplasmic expression was seen. The most common expression was diffuse cytoplasmic (73.0%, $n = 173$). 'Inside-out' expression was seen in 23 cases (9.7%), whereas 117 cases (49.4%) showed a single expression pattern. The most common single expression pattern was diffuse cytoplasmic

(70.1%, $n = 82$). One hundred cases (42.2%) showed a combination of two patterns and four (1.7%) showed a combination of three expression patterns. The most common combination of expression patterns was apical and diffuse cytoplasmic expression (40.3%, $n = 42$).

MUC1 EXPRESSION AND CLINICOPATHOLOGICAL PARAMETERS

The relationship between MUC1 expression pattern and clinicopathological parameters is shown in Table 3. Apical MUC1 expression was associated with smaller tumours ($P = 0.001$) and with lower tumour grades ($P < 0.001$).

MUC1 EXPRESSION AND BIOLOGICAL MARKERS

Table 4 shows the relationship between MUC1 expression and other biological markers.

For apical MUC1 expression a significant association with PR ($P = 0.003$) expression was found. The association with ER was not significant ($P = 0.049$). Diffuse cytoplasmic MUC1 expression showed an association with cyclin D1 ($P = 0.009$). For 'inside-out' MUC1 expression a non-significant association with ER was found ($P = 0.026$). Negativity for MUC1 was associated with ER ($P = 0.004$), PR ($P = 0.001$) and cyclin D1 ($P = 0.009$).

MUC1 EXPRESSION AND CLINICAL OUTCOME

Kaplan–Meier survival curves showed no significant correlation between MUC1 expression of the entire membrane and OS or RFS. Patients with tumours that had apical MUC1 expression displayed a better OS ($P = 0.030$; Figure 2b). No relationship between focal cytoplasmic MUC1 expression and survival was found. Patients with tumours that showed diffuse cytoplasmic MUC1 expression had a better RFS than those with tumours that did not show such expression ($P = 0.034$; Figure 2a). For 'inside-out' MUC1 expression, no correlation with survival was found. MUC1 negativity was significantly associated with worse RFS ($P \leq 0.001$) and OS ($P \leq 0.001$; Figure 2c,d).

ANALYSIS OF COMBINATIONS OF MUC1 EXPRESSION PATTERNS, CLINICOPATHOLOGICAL PARAMETERS, BIOLOGICAL MARKERS AND CLINICAL OUTCOME

In order to increase the power of the outcome analysis the expression patterns were simplified into

Table 3. MUC1 expression and clinicopathological parameters

MUC1 staining Clinicopathological parameters	Entire membrane MUC1 expression		Apical MUC1 expression		Focal cytoplasmic MUC1 expression		Diffuse cytoplasmic MUC1 expression		'Inside-out' MUC1 expression		All patterns of MUC1 immunoreactivity	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Menopausal status												
Premenopausal	16 (33.3)	59 (31.2)	19 (29.7)	56 (32.4)	3 (14.3)	72 (33.3)	54 (31.2)	21 (32.8)	7 (30.4)	68 (31.8)	67 (30.3)	8 (50.0)
Postmenopausal	32 (66.7)	130 (68.8)	45 (70.3)	117 (67.6)	18 (85.7)	144 (66.7)	119 (68.8)	43 (67.2)	16 (69.6)	146 (68.2)	154 (69.7)	8 (50.0)
(n = 237)		P = 0.778		P = 0.693		P = 0.073		P = 0.814		P = 0.895		P = 0.102
Family history												
Positive	4 (11.8)	30 (19.7)	9 (18.4)	25 (18.2)	3 (18.8)	31 (18.2)	27 (20.3)	7 (13.2)	3 (15.8)	31 (18.6)	31 (18.1)	3 (20.0)
Negative	30 (88.2)	122 (80.3)	40 (81.6)	112 (81.8)	13 (81.3)	139 (81.8)	106 (79.7)	46 (86.8)	16 (84.2)	136 (81.4)	140 (81.9)	12 (80.0)
(n = 186)		P = 0.277		P = 0.985		P = 1.000*		P = 0.259		P = 1.000*		P = 0.740*
Pathological tumour stage												
T1	17 (36.2)	89 (48.6)	39 (65.0)	67 (39.4)	11 (55.0)	95 (45.2)	77 (45.3)	29 (48.3)	9 (39.1)	97 (46.9)	98 (45.8)	8 (50.0)
T2	25 (53.2)	82 (44.8)	21 (35.0)	86 (50.6)	7 (35.0)	100 (47.6)	83 (48.8)	24 (40.0)	10 (43.5)	97 (46.9)	100 (46.7)	7 (43.8)
T3	5 (10.6)	12 (6.6)	0 (0.0)	17 (10.0)	2 (10.0)	15 (7.1)	10 (5.9)	7 (11.7)	4 (17.4)	13 (6.3)	16 (7.5)	1 (6.3)
(n = 230)		P = 0.263		P < 0.001		P = 0.548		P = 0.242		P = 0.151		P = 0.944
Tumour grade												
I	8 (16.7)	48 (25.5)	25 (39.1)	31 (18.0)	6 (30.0)	50 (23.1)	39 (22.7)	17 (26.6)	6 (26.1)	50 (23.5)	55 (25.0)	1 (6.3)
II	25 (52.1)	83 (44.1)	30 (46.9)	78 (45.3)	9 (45.0)	99 (45.8)	80 (46.5)	28 (43.8)	9 (39.1)	99 (46.5)	101 (45.9)	7 (43.8)
III	15 (31.3)	57 (30.3)	9 (14.1)	63 (36.6)	5 (25.0)	67 (31.0)	53 (30.8)	19 (29.7)	8 (34.8)	64 (30.0)	64 (29.1)	8 (50.0)
(n = 236)		P = 0.406		P < 0.001		P = 0.747		P = 0.821		P = 0.796		P = 0.113
Lymph node status												
Negative	20 (42.6)	107 (57.8)	37 (59.7)	90 (52.9)	13 (65.0)	114 (53.8)	94 (56.0)	33 (51.6)	10 (43.5)	117 (56.0)	117 (54.2)	10 (62.5)
Positive	27 (57.4)	78 (42.2)	25 (40.3)	80 (47.1)	7 (35.0)	98 (46.2)	74 (44.0)	31 (48.4)	13 (56.5)	92 (44.0)	99 (45.8)	6 (37.5)
(n = 232)		P = 0.060		P = 0.362		P = 0.335		P = 0.548		P = 0.253		P = 0.518

Table 3. (Continued)

MUC1 staining Clinicopathological parameters	Entire membrane MUC1 expression		Apical MUC1 expression		Focal cytoplasmic MUC1 expression		Diffuse cytoplasmic MUC1 expression		'Inside-out' MUC1 expression		All patterns of MUC1 immunoreactivity	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Adjuvant radiotherapy												
Yes	29 (60.4)	113 (59.8)	39 (60.9)	103 (59.5)	13 (61.9)	129 (59.7)	103 (59.5)	39 (60.9)	16 (69.6)	126 (58.9)	131 (59.3)	11 (68.8)
No	19 (39.6)	76 (40.2)	25 (39.1)	70 (40.5)	8 (38.1)	87 (40.3)	70 (40.5)	25 (39.1)	7 (30.4)	88 (41.1)	90 (40.7)	5 (31.3)
(n = 237)	$P = 0.937$		$P = 0.845$		$P = 0.846$		$P = 0.845$		$P = 0.320$		$P = 0.455$	
Adjuvant chemotherapy												
Yes	29 (60.4)	89 (47.1)	29 (45.3)	89 (51.4)	10 (47.6)	108 (50.0)	86 (49.7)	32 (50.0)	14 (60.9)	104 (48.6)	112 (50.7)	6 (37.5)
No	19 (39.6)	100 (52.9)	35 (54.7)	84 (48.6)	11 (52.4)	108 (50.0)	87 (50.3)	32 (50.0)	9 (39.1)	110 (51.4)	109 (49.3)	10 (62.5)
(n = 237)	$P = 0.099$		$P = 0.402$		$P = 0.835$		$P = 0.968$		$P = 0.263$		$P = 0.309$	

T1, Tumour diameter <20 mm; T2, tumour diameter ≥ 20 mm but <50 mm; T3, tumour diameter ≥ 50 mm.
*Fisher's exact test.

three patterns on the basis of location of MUC1 expression. Apical membrane expression and 'inside-out' expression, which were both associated with a biologically less aggressive profile, were combined with entire membrane expression and classified as membrane expression. Diffuse cytoplasmic expression was combined with focal cytoplasmic expression and classified as cytoplasmic expression. Tumours that did not show MUC1 expression were classified as MUC1–. In order to evaluate the relation between these three types of MUC1 expression and RFS and OS, the dominant type of MUC1 expression in each tumour was classified as membrane or cytoplasmic expression, or as MUC1–. In the case of multiple expression patterns in one lesion, the dominant type of expression was defined as that displayed by the largest percentage of cells.

The results of univariate Cox regression analysis for RFS are shown in Table 5. Significant relations were found for tumour size [hazard ratio (HR) 2.2, 95% confidence interval (CI) 1.1, 4.5, $P = 0.03$ for tumours between 20 and 50 mm; HR 3.8, 95% CI 1.4, 10.2, $P = 0.009$ for tumours >50 mm], tumour grade (HR 2.3, 95% CI 1.2, 4.2, $P = 0.009$), MUC1 expression (HR 3.4, 95% CI 1.5, 8.1, $P = 0.005$ for MUC1 negativity), Her2/neu expression (HR 2.8, 95% CI 1.1, 7.1, $P = 0.03$), ER expression (HR 0.5, 95% CI 0.3, 1.0, $P = 0.05$), PR expression (HR 0.4, 95% CI 0.2, 0.7, $P < 0.01$) and RFS.

Table 6 shows the results from univariate Cox regression analysis for OS. Significant results were found for tumour size (HR 6.6, 95% CI 1.6, 26.4, $P = 0.008$ for tumours >50 mm), tumour grade (HR 3.6, 95% CI 1.5, 8.7, $P = 0.005$), axillary lymph node status (HR 3.0, 95% CI 1.1, 7.8, $P = 0.03$), receipt of adjuvant chemotherapy (HR 3.2, 95% CI 1.1, 8.8, $P = 0.02$), MUC1 expression (HR 6.0, 95% CI 2.2, 16.7, $P = 0.001$), Her2/neu expression (HR 6.3, 95% CI 2.2, 17.5, $P < 0.001$), ER expression (HR 0.3, 95% CI 0.1, 0.8, $P = 0.02$), PR expression (HR 0.4, 95% CI 0.2, 1.0, $P = 0.05$) and OS.

The results from the multivariate analysis for RFS are shown in Table 7. MUC1 expression (HR 4.6, 95% CI 1.5, 8.5, $P = 0.005$ for MUC1 negativity) and PR expression (HR 0.4, 95% CI 0.2, 0.8, $P = 0.09$) were significant independent predictors of RFS.

Table 8 shows the results from multivariate analysis for OS. Axillary lymph node status (HR 4.7, 95% CI 1.7, 13.0, $P = 0.003$), MUC1 expression (HR 14.7, 95% CI 4.9, 44.1, $P < 0.001$ for MUC1 negativity) and Her2/neu expression (HR 3.7, 95% CI 1.4, 9.5, $P = 0.006$) were significant independent predictors of OS.

Table 4. MUC1 expression related to biological markers

MUC1 staining / Biological markers	Entire membrane MUC1 expression		Apical MUC1 expression		Focal cytoplasmic MUC1 expression		Diffuse cytoplasmic MUC1 expression		'Inside-out' MUC1 expression		All patterns of MUC1 immunoreactivity	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Her2/neu												
Negative	42 (87.5)	162 (86.6)	58 (92.1)	146 (84.9)	20 (95.2)	184 (86.0)	147 (85.5)	57 (90.5)	20 (87.0)	184 (86.8)	191 (86.8)	13 (86.7)
Positive	6 (12.5)	25 (13.4)	5 (7.9)	26 (15.1)	1 (4.8)	30 (14.0)	25 (14.5)	6 (9.5)	3 (13.0)	28 (13.2)	29 (13.2)	2 (13.3)
(n = 235)	P = 0.874		P = 0.150		P = 0.325*		P = 0.315		P = 1.000*		P = 1.000*	
ER												
Negative	7 (14.9)	46 (24.9)	9 (14.1)	44 (26.2)	3 (14.3)	50 (23.7)	36 (21.2)	17 (27.4)	1 (4.3)	52 (24.9)	45 (20.6)	8 (57.1)
Positive	40 (85.1)	139 (75.1)	55 (85.9)	124 (73.8)	18 (85.7)	161 (76.3)	134 (78.8)	45 (72.6)	22 (95.7)	157 (75.1)	173 (79.4)	6 (42.9)
(n = 232)	P = 0.146		P = 0.049		P = 0.422*		P = 0.316		P = 0.026		P = 0.004*	
PR												
Negative	14 (29.2)	71 (39.0)	13 (21.3)	72 (42.6)	5 (23.8)	80 (38.3)	54 (32.3)	31 (49.2)	8 (36.4)	77 (37.0)	73 (34.1)	12 (75.0)
Positive	34 (70.8)	111 (61.0)	48 (78.7)	97 (57.4)	16 (76.2)	129 (61.7)	113 (67.7)	32 (50.8)	14 (63.6)	131 (63.0)	141 (65.9)	4 (25.0)
(n = 230)	P = 0.209		P = 0.003		P = 0.190		P = 0.018		P = 0.952		P = 0.001	
p53												
Negative	46 (95.8)	176 (93.1)	62 (96.9)	160 (92.5)	19 (90.5)	203 (94.0)	165 (95.4)	57 (89.1)	21 (91.3)	201 (93.9)	209 (94.6)	13 (81.3)
Positive	2 (4.2)	13 (6.9)	2 (3.1)	13 (7.5)	2 (9.5)	13 (6.0)	8 (4.6)	7 (10.9)	2 (8.7)	13 (6.1)	12 (5.4)	3 (18.8)
(n = 237)	P = 0.491		P = 0.218		P = 0.629*		P = 0.128*		P = 0.645*		P = 0.070*	
Cyclin D1												
Negative	12 (25.0)	61 (32.3)	17 (26.6)	56 (32.4)	8 (38.1)	65 (30.1)	45 (26.0)	28 (43.8)	4 (17.4)	69 (32.2)	63 (28.5)	10 (62.5)
Positive	36 (75.0)	128 (67.7)	47 (73.4)	117 (67.6)	13 (61.9)	151 (69.9)	128 (74.0)	36 (56.3)	19 (82.6)	145 (67.8)	158 (71.5)	6 (37.5)
(n = 237)	P = 0.330		P = 0.390		P = 0.448		P = 0.009		P = 0.143		P = 0.009	

T1, Tumour diameter <20 mm; T2, tumour diameter ≥ 20 mm but <50 mm; T3, tumour diameter ≥ 50 mm.
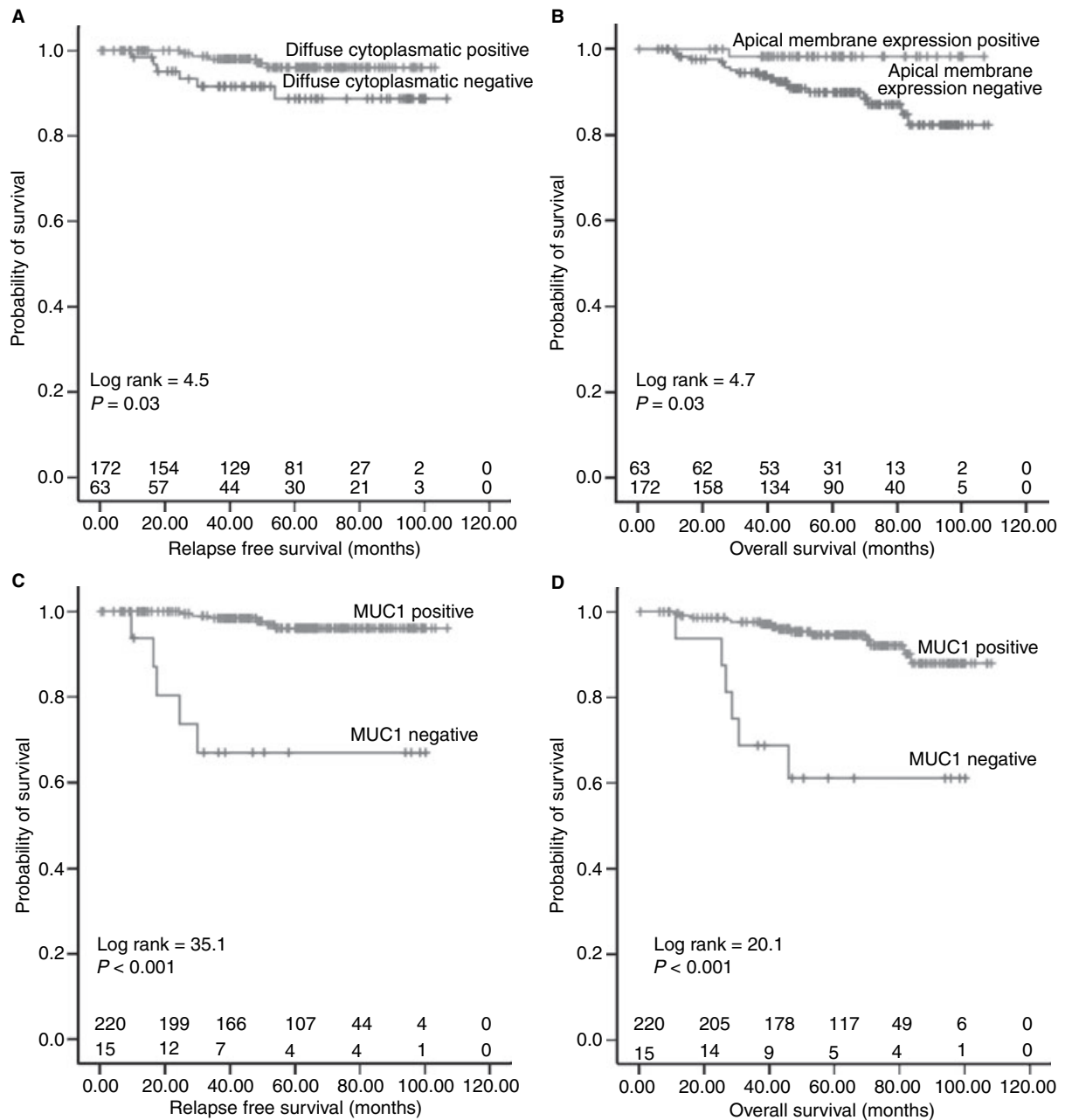*Fisher's exact test.

Figure 2. Correlation between the MUC1 expression pattern and relapse-free (**A,C**) and overall (**B,D**) survival (Kaplan–Meier method and log rank test).

Discussion

This study investigated the relationship between MUC1 expression patterns in invasive ductal carcinoma of the breast (not otherwise specified), tumour characteristics, expression of a series of well-established tumour markers and clinical outcome. To avoid ambiguous results due to the heterogeneity of breast cancer, we focused on this, by far the most common type of breast cancer.

Expression was found in 93.2% of cases. Apical MUC1 expression was significantly associated with smaller tumours, lower tumour grade, ER positivity and PR positivity. Diffuse cytoplasmic MUC1 expression showed a significant association with PR and cyclin D1 positivity. 'Inside-out' MUC1 expression was associated with ER positivity. Negativity for MUC1 was significantly associated with ER negativity, PR negativity and cyclin D1 negativity. Patients with apical

Table 5. Univariate analysis investigating the relation between pathological and biological characteristics and relapse-free survival

Pathological and biological features	n (%)	HR	95% CI	P-value
Tumour size, mm				
< 20	109 (46.2)	1		
20–50	109 (46.2)	2.2	1.1, 4.5	0.03
> 50	18 (7.6)	3.8	1.4, 10.2	0.009
(n = 236)				
Grade*				
I and II	167 (69.0)	1		
III	75 (31.0)	2.3	1.2, 4.2	0.009
(n = 242)				
Axillary lymph node status				
Negative	131 (55.0)	1		
Positive	107 (45.0)	1.6	0.9, 3.0	0.12
(n = 238)				
Adjuvant radiotherapy				
No	98 (40.3)	1		
Yes	145 (59.7)	1.1	0.6, 2.1	0.76
(n = 243)				
Adjuvant chemotherapy				
No	123 (50.7)	1		
Yes	120 (49.3)	1.4	0.7, 2.5	0.33
(n = 243)				
MUC1 expression				
Cytoplasmic	144 (60.8)			
Membrane	77 (32.5)	1	0.5, 2.0	1.0
Negative	16 (6.7)	3.4	1.5, 8.1	0.005
(n = 237)				
Her2/neu				
Negative	225 (94.5)	1		
Positive	13 (5.5)	2.8	1.1, 7.1	0.03
(n = 238)				
ER				
Negative	54 (22.9)	1		
Positive	182 (77.1)	0.5	0.3, 1.0	0.05
(n = 236)				

Table 5. (*Continued*)

Pathological and biological features	n (%)	HR	95% CI	P-value
PR				
Negative	86 (36.9)	1		
Positive	147 (63.1)	0.4	0.2, 0.7	0.003
(n = 233)				
p53				
Negative	226 (93.8)	1		
Positive	15 (6.2)	1.5	0.5, 4.3	0.5
(n = 241)				
Cyclin D1				
Negative	75 (30.9)	1		
Positive	168 (69.1)	0.6	0.3, 1.2	0.2
(n = 243)				

n, Number of cases; HR, hazard ratio; ER, oestrogen receptor; PR, progesterone receptor.
*Because of small numbers, grades I and II were combined.

MUC1-expressing tumours and patients with diffuse cytoplasmic MUC1-expressing tumours displayed a significantly increased RFS. Patients with tumours negative for MUC1 showed a significantly decreased RFS and OS on both univariate and multivariate analysis.

Before discussing the associations found for the different expression patterns of MUC1 in more detail, it is important to discuss the antibodies that have been used to detect MUC1 in various studies. Almost all anti-MUC1 antibodies used are directed against the O-glycosylated extracellular MUC1 tandem repeat domain.[13] However, the degree and make-up of glycosylation may vary extensively among MUC1+ adenocarcinomas,[25,26] and the affinity for MUC1 of the vast majority of these antibodies depends on the extent and composition of glycosylation.[27,28] As a consequence, the variety of anti-MUC1 antibodies used to determine MUC1 expression in breast carcinoma may explain at least some of the discrepancies between various studies, as discussed below.

Detecting almost all glycosylated MUC1 isoforms is important in studying its significance for tumour progression, relationship to other tumour progression markers and to clinical outcome. Some well-established functions of MUC1, e.g. inhibition of cell–cell and cell–extracellular matrix adhesion, are only to a minor

Table 6. Univariate analysis of the relation of pathological and biological characteristics with overall survival

Pathological and biological features	n (%)	HR	95% CI	P-value
Tumour size, mm				
< 20	109 (46.2)	1		
20–50	109 (46.2)	3.0	1.0, 9.2	0.06
> 50	18 (7.6)	6.6	1.6, 26.4	0.008
(n = 236)				
Grade*				
I and II	167 (69.0)	1		
III	75 (31.0)	3.6	1.5, 8.7	0.005
(n = 242)				
Axillary lymph node status				
Negative	131 (55.0)	1		
Positive	107 (45.0)	3.0	1.1, 7.8	0.03
(n = 238)				
Adjuvant radiotherapy				
No	98 (40.3)	1		
Yes	145 (59.7)	1.0	0.4, 2.4	0.95
(n = 243)				
Adjuvant chemotherapy				
No	123 (50.7)	1		
Yes	120 (49.3)	3.2	1.1, 8.8	0.02
(n = 243)				
MUC1 expression				
Cytoplasmic	144 (60.8)	1		
Membrane	77 (32.5)	0.7	0.2, 2.3	0.6
Negative	16 (6.7)	6.0	2.2, 16.7	0.001
(n = 237)				
Her2/neu				
Negative	225 (94.5)	1		
Positive	13 (5.5)	6.3	2.2, 17.5	< 0.001
(n = 238)				
ER				
Negative	54 (22.9)	1		
Positive	182 (77.1)	0.3	0.1, 0.8	0.02
(n = 236)				

Table 6. (Continued)

Pathological and biological features	n (%)	HR	95% CI	P-value
PR				
Negative	86 (36.9)	1		
Positive	147 (63.1)	0.4	0.2, 1.0	0.05
(n = 233)				
p53				
Negative	226 (93.8)	1		
Positive	15 (6.2)	2.4	0.7, 8.1	0.2
(n = 241)				
Cyclin D1				
Negative	75 (30.9)	1		
Positive	168 (69.1)	0.6	0.2, 1.5	0.3
(n = 243)				

n, Number of cases; HR, hazard ratio; ER, oestrogen receptor; PR, progesterone receptor.
*Because of small numbers, grades I and II were combined.

Table 7. Stepwise multivariate analysis investigating the relation of pathological and biological characteristics with relapse-free survival

Characteristics	HR	95% CI	P-value
MUC1 expression			
Cytoplasmic	1		
Membrane	1.1	0.5, 2.2	0.8
Negative	3.5	1.5–8.5	0.005
PR			
Negative	1		
Positive	0.4	0.2, 0.8	0.09

HR, Hazard ratio; 95% CI, 95% confidence interval.

extent dependent on MUC1 glycosylation status.[7,29] For that reason, we used mAb 214D4, a monoclonal antibody which is also directed to the protein backbone of the MUC1 repeat domain, but for which affinity is almost independent of glycosylation status.[21]

In normal glandular epithelium, MUC1 is expressed at the apical surface.[5] For that reason, apical expression in breast carcinomas (designated 'pattern B', Figure 1) indicates normal routing of MUC1 molecules and, as a consequence, relatively intact glandular

Table 8. Stepwise multivariate analysis investigating the relation of pathological and biological characteristics with overall survival

Characteristics	HR	95% CI	P-value
Axillary lymph node status			
Negative	1		
Positive	4.7	1.7. 13.0	0.003
MUC1 expression			
Cytoplasmic	1		
Membrane	0.6	0.2, 2.0	0.4
Negative	14.7	4.9, 44.1	< 0.001
Her2/neu			
Negative	1		
Positive	3.7	1.4, 9.5	0.006

HR, Hazard ratio; 95% CI, 95% confidence interval.

differentiation. Indeed, in our series, apical MUC1 expression was associated with many indicators of good prognosis and a better OS. The association with lower tumour grade,[11,13,15,30] ER[15,30] and PR[30] positivity and the absence of distant metastasis[15] has been described. Some authors have found an increased rate of axillary lymph node negativity[15] and longer RFS for patients with tumours showing apical MUC1 expression.[15,31] Our data did not confirm these findings. Study size, follow-up and the patients included might account for this difference, e.g. the study by Hayes *et al.* included only node-positive patients. In accordance with our series, an increase in OS of patients with tumours showing apical MUC1 expression has been reported elsewhere.[15,32] One relatively small study found no association between apical expression and clinicopathological variables.[33]

Entire membrane MUC1 expression (designated 'pattern A', Figure 1) is more often seen in mucinous carcinomas than in ductal carcinomas of no special type.[12] Although this expression pattern appears to be the effect of misrouting in the MUC1 pathway, no unambiguous results on the role of MUC1 expression on the entire membrane in breast cancer have been described. Whereas Parham *et al.* have shown that high entire membrane expression of MUC1 is associated with low tumour grade,[17] Rahn *et al.* have shown the opposite.[13] The former study also found an association with positive lymph node status. In the current study no significant associations between MUC1 entire membrane expression and clinicopathological parameters were found. Entire membrane MUC1 expression did not

associate with clinicopathological characteristics and outcome in these series. Two other studies that have looked at the relationship between expression of MUC1 on the entire membrane and outcome have also found no such relation.[11,17] By combining entire membrane and cytoplasmic MUC1 expression, Rakha *et al.* were able to show a significant decrease in OS and RFS in this group.[15] We did not perform such a subgroup analysis.

'Inside-out' expression (designated 'pattern E', Figure 1) for MUC1 was present in a small percentage of the tumours and has been previously described by two of the authors (C.P., J.L.P.).[34] This pattern is specific for invasive micropapillary carcinoma, a subtype of ductal breast carcinoma with a high potential to metastasize to axillary lymph nodes.[35] We found no such relationship, however, nor did we find an association between 'inside out' expression and outcome. The small number of cases in these series might account for this.

Diffuse cytoplasmic expression of MUC1 was associated with good prognosis in these series. Previous reports have linked cytoplasmic expression of MUC1 to ER negativity,[15] high Her2/neu expression,[36] decreased RFS[11,15,32] and decreased OS.[15,32] The study by Lundy *et al.* found that MUC1 cytoplasmic expression was related to ER positivity and lower tumour grade.[33] In this study a positive relationship between MUC1 diffuse cytoplasmic expression and PR and cyclin D1 positivity was found, which might be explained by the common combination of apical and diffuse cytoplasmic expression in these series. Results from the subgroup analysis of combined apical and diffuse cytoplasmic MUC1 expression versus strictly cytoplasmic MUC1 expression show that tumours with diffuse cytoplasmic MUC1 expression have a clinicopathological profile that is usually associated with a worse outcome, but that when this is combined with apical MUC1 expression (i.e. a part of the MUC1 is routed correctly) this negative effect disappears.

Focal cytoplasmic expression of MUC1 has been described in lobular carcinoma.[12,37] To our knowledge, it has not previously been described in ductal carcinoma. We did not find any relationship between focal cytoplasmic expression (designated 'pattern C') and any of the investigated variables.

We observed that tumours negative for MUC1 had a very poor outcome with respect to RFS and OS (Figure 2C,D). In addition, absence of MUC1 expression was associated with absence of ER, PR and cyclin D1. These findings support the observation by Luna-More *et al.* that tumours negative for MUC1 are high grade, are ER– and PR– and are more frequently associated with positive axillary lymph nodes.[30] Other studies

have related low or negative MUC1 expression to higher tumour grade[13] and poor prognosis.[38] Inflammatory breast carcinoma patients with MUC1– tumours had a significantly shorter OS.[39] Remarkably, our MUC1– group of breast carcinomas appears to be a subgroup with poor prognosis that cannot be identified with the common prognostic indicators; for both RFS and OS survival MUC1 negativity was the strongest independent predictor (see Tables 7 and 8).

We also performed a study with MUC1 expression in DCIS (unpublished results). Comparison of those results with the current study reveals some interesting differences. The 'inside-out' expression pattern is exclusively seen in invasive ductal carcinomas and not in DCIS. Also in DCIS, no MUC1– tumours were found. As mentioned before, the 'inside-out' expression pattern is specific for invasive micropapillary carcinoma. MUC1– tumours are a subgroup of tumours that is non-luminal, non-mucin producing. These tumours are probably fast growing and aggressive and may not have a stage of non-invasive growth that can be easily identified because of early invasion. Loss of MUC1 might play a role in this process of early invasiveness. Remarkably, this seems to be in contrast to *in vitro* and *in vivo* data, which show that membranous MUC1 overexpression favours adhesion modulation, invasive potential and metastatic capacity of tumour cells.[6–9] These effects are very likely due to steric hindrance of adhesion molecules by the high density of large and elongated extracellular MUC1 domains at the cell surface.[7] Undoubtedly, there are more mechanisms available for acquiring invasive potential, e.g. inactivation of the E-cadherin–β-catenin complex as in invasive lobular breast carcinoma. To investigate a potential relationship between MUC1 and E-cadherin expression, we performed immunohistochemistry for E-cadherin and β-catenin. However, the immunoreactivity of both proteins was too heterogeneous and inconsistent for reliable semiquantitative analysis (data not shown).

In this outcome study, patients were treated in a very heterogeneous manner (radiotherapy, chemotherapy and hormonal therapy) and this may have confounded the results somewhat. However, on univariate analysis radiotherapy is not a predictor for either OS or RFS. On multivariate analysis MUC1 negativity remains an independent predictor of RFS and OS, suggesting an effect independent of adjuvant therapy. Because of the many comparisons presented in Tables 3 and 4, only *P*-values (uncorrected) < 0.01 are presented. It should be noted that this part of the study was of an exploratory nature, based on the hypothesis that MUC1 expression patterns provide added value in relation to clinicopathological parameters. In addition, the selection of such clinicopathological parameters and biomarkers was based on their established role in the biology of carcinomas and in general breast carcinoma in particular. Therefore, it is hoped that the conclusions of this study will contribute to the optimal determination of the clinical impact of MUC1 expression in invasive ductal breast carcinoma.

Acknowledgement

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