MUC1 (episialin) expression in non-small cell lung cancer is independent of EGFR and c-erbB-2 expression and correlates with poor survival in node positive patients

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Abstract

Aim—To examine tumour samples immunohistochemically for MUC1 (episialin), epidermal growth factor receptor (EGFR), and c-erbB-2, since the disruption of the cell-cell adhesion system by MUC1 and the c-erbB oncoprotein family is known to be important in the development of metastasis in human cancers.

Methods—93 tumour samples from patients with early stage non-small cell lung cancer treated with surgery alone were examined for episialin, EGFR, and c-erbB-2.

Results—Episialin depolarised expression did not correlate with any of the histopathological variables examined (T, N stage, grade, histology, Ki67 proliferation index). No correlation was observed between episialin and EGFR or c-erbB-2 expression. Survival analysis showed that episialin depolarised expression correlated with poor prognosis ($p = 0.003$), especially in squamous cell cases ($p = 0.0003$). Episialin expression defined a group of patients with poor prognosis in the node positive category ($p = 0.003$). In multivariate analysis episialin was the most significant independent prognostic factor ($p = 0.007$), followed by N stage ($p = 0.04$).

Conclusions—Depolarised expression of episialin is associated with poor outcome in early stage non-small cell lung cancer. Despite the similar activity on the cadherin cell-cell adhesion system, the expression of episialin and c-erbB oncoproteins is likely to be activated within different pathogenic pathways.

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For a cancer to invade adjacent structures and to migrate to distant organs, complex mechanisms are required. Angiogenic factor secretion by tumour and stromal cells, endothelial cell migration and vessel formation, and expression of proteins involved in the cell-cell and cell-matrix adhesion disruption or cell motility are essential for the tumour to grow, invade, and metastasise.^{1,2}

The cadherin-catenin cell-cell adhesion system is well known to be involved in animal

morphogenesis and the maintenance of normal adhesion between cells.³ The epidermal growth factor receptor (EGFR) and c-erbB-2 transmembrane oncoproteins (members of the erb oncoprotein family) interact with the cadherin-catenin complex and may inhibit cell adhesion function, thus enhancing cancer cell invasive potential.^{4,5} Episialin, also known as MUC1 (or PEM, CA-15-3 antigen, or EMA) is another transmembrane protein shown in vitro to reduce E-cadherin mediated cell-cell adhesion by steric hindrance.⁶ Episialin is a glycoprotein expressed at the apical side of normal glandular epithelial cells. In cancer cells, depolarised expression throughout the entire cell surface has been observed.⁷

In this study we examined the depolarised expression of MUC1 glycoprotein immunohistochemically in a series of early stage operable non-small cell lung cancers. We report correlations with histopathological variables, Ki67 mitotic index, EGFR, and c-erbB-2, as well as survival.

Methods

We examined 93 tumour surgical samples from patients with early operable (T1,2-N0,1 staged⁸) non-small cell lung cancer. All patients were treated with surgery alone, without radiotherapy or chemotherapy. Histological diagnosis, grading, and N stage was done on haematoxylin and eosin stained sections. Of these, 35 were adenocarcinomas and 58 squamous cell lung carcinomas. Follow up ranged from 45 to 74 months (median 62).

EPISIALIN IMMUNOHISTOCHEMISTRY

Episialin MUC1 expression was assessed on paraffin embedded material with the 214D4 MoAb (IgG1) using an avidin-biotin complex immunoperoxidase technique. This antibody recognises the protein backbone of episialin. Sections were dewaxed and rehydrated, and treated for 10 minutes with 3% H₂O₂ to limit endogenous peroxidase activity. Samples were then washed three times in 50 mM Tris-HCl/150 mM NaCl, pH 7.6 (TBS), and non-specific binding was blocked in normal rabbit serum (30 minutes, 1:20; Dako, Copenhagen, Denmark) in TBS, and incubated with the monoclonal antibody 214D4 (mouse IgG1, diluted 1:32 000) overnight at 4°C. The sections were washed thoroughly in TBS and incubated with biotin conjugated rabbit

Figure 1 (A) A lung adenocarcinoma with polarised MUC1 expression. (B) A lung squamous cell carcinoma with depolarised circumferential cytoplasmic and membranous MUC1 expression.

antimouse immunoglobulin antibody (30 minutes, 1:200, Dako), followed by an avidin–biotin–peroxidase complex (30 minutes, 1:100, Dako). Finally, the sections were incubated with 3–4–3–4–diaminobenzidine as chromogen for five minutes and counterstained with haematoxylin. Omission of the primary antibody was used for negative control.

Figure 2 Linear regression of the percentage of deaths in sequential groups of patients defined by increasing cut off percentages of depolarised episialin expression in 93 non-small cell lung cancer patients.

In normal epithelium, episialin shows a polarised pattern of immunoreactivity. Positive “polarised pattern” of expression is defined when episialin is localised in the cytoplasmic vacuoles or in the cell membrane at the apical site of cells. The circumferential cytoplasmic and membrane immunoreactivity, never seen in normal cells, was recorded as “depolarised” episialin expression (fig 1). The percentage of cancer cells with episialin depolarised expression was recorded. This allowed analysis using MUC1 as a continues variable. Cases were also divided into two groups following the percentage of cells with depolarised expression, thus positive (depolarised expression in more than 25% of cells) and negative (0–25%). The 25% used cut off point was the mean percentage of cells with episialin depolarised expression, but also the point that defined the group of patients with the highest percentage of deaths at the time of analysis (fig 2).

OTHER IMMUNOHISTOCHEMICAL STUDIES
C-erbB-2 oncoprotein expression was assessed with the monoclonal antibody NCL-CB11 (Novocastra Laboratories, Newcastle upon Tyne, UK), which recognises the internal domain of the c-erbB-2 protein amino acid sequence. Staining was done with a previously described indirect immunoperoxidase technique.⁹ For a positive control we used a breast carcinoma with 15-fold amplification of the c-erbB-2 gene and as a negative the primary antibody was omitted. In a previous study we showed that membrane staining was impossible to assess in non-small cell lung cancer.⁹ C-erbB-2 expression was separately assessed for cytoplasmic and membrane patterns of reactivity. Two groups were considered for cytoplasmic staining: the group with positive reactivity (strong staining intensity in

Table 1 Correlation between episialin depolarised expression and histological and patient variables in 93 non-small cell lung cancer cases

Variable		Mean % positive cells (SD)	95% CI	p Value
Histology	SC	31.2 (31)	23 to 39	0.81
	AC	29.7 (30)	19 to 40	
T stage	T1	33.8 (31)	22 to 45	0.48
	T2	29.0 (30)	21 to 36	
N stage	N0	30.3 (30)	22 to 38	0.88
	N1	31.2 (32)	20 to 42	
Grade	I/II	28.0 (32)	18 to 37	0.44
	III	32.9 (29)	24 to 41	
Ki67	L/M	31.2 (31)	24 to 38	0.67
	H	27.2 (29)	12 to 43	
c-erbB-2	neg/weak	28.5 (31)	21 to 35	0.31
	pos	35.9 (29)	23 to 48	
EGFR	neg	36.6 (36)	18 to 54	0.57
	pos	31.7 (30)	23 to 39	
Age	<65 years	32.2 (32)	23 to 41	0.55
	>64 years	28.4 (28)	19 to 37	
Sex	Female	38.5 (32)	22 to 54	0.23
	Male	28.8 (30)	21 to 35	

AC, adenocarcinoma; CI, confidence interval; SC, squamous cell carcinoma.

Figure 3 Kaplan-Meier survival curves for all cases (A), squamous cell cases (B), and adenocarcinomas (C) according to MUC1 depolarised expression (positive v negative).

> 50% of cells) and the group with negative or weak reactivity (all other cases). Membrane reactivity was positive when more than 10% of cells had a clearly defined membrane staining.

EGFR1 murine monoclonal antibody was used to identify EGFR by means of an indirect immunoperoxidase technique¹⁰ on 5 µm cryostat sections. Samples with negative (–) or weak (+) staining intensity were considered as negative, while moderately (++) or strongly (+++) positive were considered as positive.

Proliferative index was assessed with the monoclonal antibody Ki67.¹¹ Frozen material was taken from two separate areas of the tumour and the Ki67 assessment was based on the average value. Three groups were considered, based on the percent of stained nuclei: 0–10% = low proliferative index, 10–40% = medium, and > 40% = high.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Stata 3.1 Package (Stata Corporation, Texas, USA). An unpaired two tailed *t* test was used for testing relations between categorical tumour variables. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumour variables on overall survival. A probability (*p*) value < 0.05 was considered significant.

Results

Depolarised MUC1 expression in more than 25% of cancer cells was observed in 40 of 93 cases (43%), where 28 of 58 squamous cell carcinomas (48%) and 12 of 35 adenocarcinomas (34%) showed depolarised patterns. Table 1 shows the correlation of MUC1 depolarised expression with histological and patient variables in 93 non-small cell lung cancer cases. No association was found with histology, nodal metastases, tumour differentiation, Ki67 proliferation index, patient age, or sex. No correlation of MUC1 with EGFR or cytoplasmic c-erbB-2 expression was observed. Analysis for membrane c-erbB-2 reactivity was impossible since only three of 93 cases (3.2%) showed a clearly defined pattern of membrane staining.

Overall survival analysis showed that MUC1 depolarised expression was associated with a significantly worse prognosis (*p* = 0.003; fig 3A). This mainly concerned the cases with squamous cell histology (*p* = 0.0003; fig 3B), while no significant association with survival was observed in the adenocarcinomas (*p* = 0.7; fig 3C). Analysis within the N stage groups showed that MUC1 depolarised expression defined a group of node positive cases with significantly worse prognosis (fig 4, A and B; *p* = 0.009). Multivariate analysis showed that MUC1 expression was the most significant independent prognostic factor (*b* = 0.85, SE = 0.30, *p* = 0.004) followed by N stage (*b* = 0.62, SE = 0.30, *p* = 0.04). None of the remaining variables had an independent prognostic significance.

Figure 4 Kaplan-Meier survival cases for N0 stage (A) and N1 stage (B) groups according to MUC1 depolarised expression (positive v negative).

Discussion

Several *in vitro* studies suggest that MUC1 expression by cancer cells is an important component of biochemical events that enable metastasis. Wesseling *et al* showed that MUC1 inhibits the E-cadherin mediated cell-cell adhesion system, the length of MUC1 ectodomain being the dominant factor for inhibition to occur.⁶ High MUC1 levels also reduce the integrin mediated cell adhesion to the extracellular matrix.¹² Although non-restricted T cell cytotoxicity to the major histocompatibility complex elicited by MUC1 is reported,^{13, 14} MUC1 expressing cells are less susceptible to T cell mediated lysis, which may contribute to cancer cell escape from immune surveillance.¹⁵

In this study we examined immunohistochemically the depolarised expression of MUC1 in a series of non-small cell lung cancer patients treated with surgery alone. Depolarised MUC1 expression in more than 25% of cells was observed in about 45% of cases. High levels of MUC1 mRNA in lung cancer have been recently reported.¹⁶ In a study by Nguyen *et al*,¹⁷ high concentrations of MUC1 mRNA

were found in normal lung tissue and adenocarcinomas, while MUC1 levels were not increased in squamous cell cancer. In our study we examined the depolarised expression of MUC1 and found no difference between squamous cell carcinomas and adenocarcinomas. Disturbance of localisation rather than the mRNA expression level may more accurately reflect the aberrant function of the MUC1 membrane bounding mucin.

We also observed no correlation between MUC1 expression in lung cancer and histopathological variables including N stage, differentiation, and proliferation index. In breast cancer,¹⁸ depolarised MUC1 expression has been associated with node involvement and positive oestrogen receptors. However, no correlation of MUC1 with T stage or grade was observed. A significant association between MUC1 expression and node involvement has also been reported in colorectal cancer.¹⁹

The c-erb protein family is well known to disrupt the cadherin mediated cell adhesion system. In three studies of non-small cell lung cancer, c-erbB-2 expression was found to be associated with a worse outcome.²⁰⁻²² In a previous study we showed that c-erbB-2 defined a poorer survival in cases with low vascularisation.²³ Although membrane staining is generally accepted to be functionally relevant to tumour pathogenesis, only Kern's study²⁰ was based on membrane staining. The studies by Tateishi *et al*,²¹ Harpole *et al*,²² and Giatromanolaki *et al*²³ did not recognise a clearly defined pattern of membrane staining, the whole analysis been founded on cytoplasmic reactivity. Moreover, in a study by Kay *et al*,²⁴ although membrane positivity was found in 7-21% of breast, bladder, and renal cases, no membrane reactivity was identified in squamous cell carcinomas, colon adenocarcinomas, and other tumours. Thus it seems that assessment of membrane reactivity cannot always be applied. Only 3.2% of our cases expressed a clearly defined pattern of c-erbB-2 membrane staining. Further staining of our cases with another c-erbB-2 antibody (Dako) using the APAAP technique confirmed that membrane staining assessment is not feasible in non-small cell lung cancer (data not shown). In the present study we examined possible cooperation of the MUC1 protein with the EGFR and c-erbB-2 oncoproteins. Although an unpublished study reported downregulation of MUC1 after transfection of mammary cancer cells with c-erbB-2,²⁵ we failed to confirm a similar association. This may show that in lung cancer, although MUC1 and c-erbB proteins mediate cell-cell adhesion disruption, the regulation of their expression is controlled by different mechanisms.

Although several reports show a correlation of MUC1 expression with survival in breast, colon, and prostate cancer,^{18, 26, 27} the role of MUC1 expression in lung cancer has not been clarified. A preliminary report from Japan in 38 non-small cell lung cancer cases revealed a possible association of MUC1 expression with poor survival.²⁸ In our study, despite the absence of MUC1 association with

histopathological variables, we observed a striking association of MUC1 depolarised expression with poor survival in node positive non-small cell lung cancer cases. Up to now nodal metastasis is considered the most important prognostic variable, and this was also confirmed in a previous study of our own.²⁹ This is probably because node involvement is the best proof of cancer cell ability to migrate and form colonies. Why MUC1 expression in node positive cases correlates with poorer survival requires further investigation. Escape from immune surveillance¹⁵ could be an explanation, although a possible role of MUC1 in pure haematogenous spread cannot be excluded. Indeed, the interaction between MUC1 and ICAM-1 has recently been reported to be critical for the expression of bloodborne metastases in breast cancer.³⁰ Our findings further support the existence of differences between pathogenic pathways that control lymphatic and bloodborne metastases.

We conclude that MUC1 is an important molecule, the depolarised expression of which relates to poor outcome in early stage non-small cell lung cancer. Although both MUC1 and c-erbB oncoproteins are involved in the disruption of cell-cell adhesion, it seems that their upregulation is triggered by non-overlapping mechanisms which are activated within different pathogenic pathways.

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