Potential prognostic value in human breast cancer of cytosolic Nme1 protein detection using an original hen specific antibody

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Summary The metastasis-suppressor *nme* gene (also called *nm23*), first identified in murine melanoma cells, exists as two forms in human: *nme1* and *nme2*. However, only the lack of expression of *nme1* has been related to distant metastasis appearance in human breast cancer. The aim of this work was first to raise specific antibodies to allow the analysis of Nme1 and then, with this specific tool, to evaluate the predictive value of Nme1 detection in cytosolic extracts of human breast tumours. We obtained a hen antibody that specifically reacts with Nme1 without any cross-reaction with Nme2. We analysed the expression of the protein in 59 human breast tumours and found a significant relationship between this expression and oestrogen receptor status ($P<0.001$). Moreover, Nme1 expression is related to metastasis-free survival ($P<0.001$) and survival of patients ($P<0.001$). The determination of Nme1 expression in primary tumours using our antibody should be an interesting predictive test of the metastasis for clinical investigations.

Keywords: metastasis-suppressor gene *nme1*; Nme1 specific antibody; Western blotting analysis; human breast tumour

The metastasis-suppressor gene, *nme1* was identified in 1988 (Steeg *et al.*, 1988) by differential hybridisation between low and high metastatic murine melanoma cell lines: the mRNA expression of this gene was found to be higher in the low metastatic cell line. In human, the *nme* gene family consists of two closely related genes: *nme1* and *nme2*, which respectively code for two different subunits (A and B) of nucleotide diphosphate kinases (NDP kinases) (Gilles *et al.*, 1991). These two subunits, which show more than 88% homology, associate together in different ratios according to tissue location, to form hexameric NDP kinases. This class of enzymes is involved in microtubule association (Nickerson and Wells, 1991) and G-protein regulation (Kimura and Shimada, 1988) but the precise molecular mechanism underlying the role of *nme* genes in metastasis dissemination is still unclear. The metastasis-suppressor activity of *nme1* seems to be correlated more with serine phosphorylation of Nme1 protein (MacDonald *et al.*, 1993) than with kinase activity (Sastre-Garau *et al.*, 1992). A recent report (Howlett *et al.*, 1994) suggests that *nme1* may play an important role in the differentiation of human breast cancer. Moreover, Postel *et al.* (1993) has suggested that the Nme2 protein is a transcription factor for the c-myc oncogene.

The predictive value of *nme* detection has been evaluated by several authors in human tumours. Reduced expression of *nme1* has been found in ovarian carcinomas (Mandai *et al.*, 1994), in hepatocellular carcinoma (Nakayama *et al.*, 1992; Yamaguchi *et al.*, 1994; Boix *et al.*, 1994), in metastatic lymph nodes from patients with papillary carcinoma of the thyroid (Arai *et al.*, 1993) and in metastasis of malignant melanoma (Xerri *et al.*, 1994). Conflicting results have been obtained in colorectal tumorigenesis: Yamaguchi (1993) obtained lower amounts of *nme1* mRNA and protein in colorectal tumours associated with liver metastasis than in those without such metastasis while Myeroff *et al.* (1993) showed an increase of the mRNA level in both high- and low-metastatic potential tumours, suggesting that the activity of the *nme* gene could be tissue specific. In breast cancer a

low level of *nme1* mRNA has been correlated with a high metastatic potential (Henessy *et al.*, 1991; Bevilacqua *et al.*, 1989) to a greater extent than *nme2* (Stahl *et al.*, 1991).

The *nme1* gene is frequently affected by loss of heterozygosity (LOH) (Leone *et al.*, 1991) but *nme1* LOH was not uniformly associated with low protein expression and poor prognosis (Cropp *et al.*, 1994). Studies using antibodies directed against NDP kinase A have been performed to measure Nme1 protein expression in tumours but, probably because of the high sequence homology between NDP kinase A and B, and the poor predictive value of the B form, these studies produced controversial results (Sawan *et al.*, 1994; Lacombe *et al.*, 1991). The first aim of the current work was to raise an antibody that would specifically recognise Nme1 protein without any cross-reaction with Nme2, to allow specific analysis of Nme1 expression. With this tool, we could then evaluate the predictive value of Nme1 expression: for this, we studied the relationship between Nme1 expression and other prognostic factors such as menopausal status, tumour size, histological grade, lymph node status, hormone receptors, cathepsin D expression, pS2, c-erbB-2. We then related Nme1 expression to disease-free survival and overall survival in patients.

Materials and methods

Normal and tumour tissues

Breast biopsy specimens were obtained from 59 patients with operable primary breast cancer who entered the Claudius Regaud Center between August 1989 and November 1990. The samples, collected after surgical removal, were stored in liquid nitrogen until use. Four specimens of normal breast tissue obtained from patients following mammary reduction were similarly treated.

The patients entering this study met the following criteria: primary breast cancer, availability of complete clinical, histological and biological information, no other primary cancer and no preliminary treatment. Patients underwent physical examination every 3 months for the first 2 years and every 6 months thereafter. Post-surgical treatment was planned according to prognostic factors and included chemotherapy, hormonetherapy and/or radiotherapy.

Pathology

Tumour size was recorded as the largest diameter of the tumour. Modified Scarff, Bloom and Richardson grades (Contesso et al., 1987) were assigned to all tumours by scoring tubular formation, nuclear pleomorphism and mitosis. Lymph node status was sought and histological examination used to confirm the involvement with tumour cells. The average number of lymph nodes examined was 15 and 30% of the patients showed no lymph node invasion.

Synthesis of peptide antigen

The peptide sequence of the antigen was designed from the amino acid sequence of NDP kinase A (Stahl et al., 1991) to be divergent with the corresponding sequence of NDP kinase B: the 16 amino acid peptide from glycine 37 to tyrosine 53 of the protein was synthesised by Novabiochem (France), purified by high-performance liquid chromatography (HPLC) and coupled to haemocyanine to increase its immunoreactivity.

Immunisation of the hen and antibody extraction

The hen received a first injection in the peritoneal muscle of 10 μg of haemocyanin–peptide complex dissolved in 500 μl of complete Freund's adjuvant (Calbiochem, France). Two identical injections were then given 12 and 20 days later (Gassmann et al., 1990). The eggs were collected daily and stored at 4°C until processing. Egg yolk was separated from the white, carefully washed with deionised water to remove albumin, diluted in 60 ml of a 3.5% polyethylene glycol solution (PEG 6000, Pharmacia, France), and centrifuged for 10 min at 14 000 g at 4°C. The supernatant was filtered and adjusted to 9% PEG 6000; the solution was centrifuged for 10 min at 9000 g at 4°C. The pellet was resuspended in 40 ml of 12% PEG and centrifuged for 10 min at 9000 g at 4°C. The final pellet was resuspended in 5 ml of phosphate-buffered saline (PBS) and stored at −20°C until use. In this solution, the IgY represented more than 80% of the total protein extracted and the specific antibody against NDP kinase A was 50% of the total IgY (Gassmann et al., 1990). Protein concentration was measured according to the Bradford technique (Bradford, 1976).

Analysis of antibody specificity

The labelling specificity of the egg yolk antibody was analysed by Western blot. NDP kinase A and B, purified as described previously (Gilles et al., 1991), were kindly provided by Dr Lascu (University of Bordeaux 2, France).

Extraction of cytosols and membranes from tumours

The breast tumour or normal breast tissue was homogenised in 10 mM Tris buffer pH 7.4 containing 20 mM molybdic acid, 12 mM monothioglycerol and centrifuged at 105 000 g for 30 min at 4°C. The supernatant represented the cytosolic extract. The pellet was resuspended in 5 mM Hepes, 3 mM EDTA, 1% Triton X100, 4 mM benzamidine, 1 mM DTT pH 7.4, and centrifuged for 30 min at 105 000 g. This supernatant was the membrane extract. Protein concentrations were determined using the Bradford technique (Bradford, 1976) for cytosolic extract and bicinchoninic acid (Smith et al., 1985) for the membrane extract.

Measurement of oestrogen or progesterone receptors, cathepsin D and pS2

The amount of hormonal receptors in the cytosolic extracts was determined using oestrogen receptor and progesterone receptor EIA kits (Abbott, France). The cathepsin D and pS2 cytosolic protein amounts were quantified using specific kits (CIS France).

Measurement of the amount of erbB-2 protein

Total protein (50 μg) from membrane extracts were diluted in 10 mM Tris HCl, 150 mM sodium chloride, 0.2% Tween20 pH 8 (TBST) and spotted onto nitrocellulose membrane (0.2 μm Biorad) under vacuum (Dot Blot system, Biorad, France). The membrane was then incubated at room temperature in TBST containing 1% lyophilised milk powder for 90 min and then for 1 h with the same solution containing the monoclonal antibody c-neu Ab3 (Oncogene Science, France) at 10 μg ml^{-1}. After three successive washes in TBST the nitrocellulose membrane was incubated in TBST containing 0.75 μg ml^{-1} of peroxidase-conjugated goat anti-mouse antibody (Tebu, France) and washed again three times with TBST. After incubation in the ECL system (Amersham, France), the membrane was exposed for 5 s to Hyperfilm MP (Amersham, France). The film was scanned using a densitometer (Performance, Sebia, France). Variable amounts (from 10 μg to 60 μg) of protein extracted from MCF 7 membranes under the same conditions as the tumours, were loaded onto the same membrane. The signal was quantified using the densitometer and the calibration curve was then drawn. The amount of ErbB-2 protein contained in tumours was determined from this curve and expressed in arbitrary units determined as equivalent μg MCF 7 membrane protein per μg of tumour protein loaded.

Western blot analysis of Nme1

Total cytosolic protein (60 μg) from each sample and 1 μg of pure NDP kinase (Sigma, France) were loaded on a 15% polyacrylamide gel and electrophoresed at 150 V as described by Laemmli (1970). The gel was equilibrated in transfer buffer (480 mM Tris pH 8, 390 mM glycine, 0.5% sodium dodecyl sulphate (SDS), 25% methanol) for 10 min at room temperature. The transfer was then performed on a 0.2μm nitrocellulose membrane at 250 mA for 2 h in the transfer buffer. The membrane was incubated in TBST containing 1% lyophilised milk powder for 90 min, then in the same solution containing extracted anti-Nme1 IgY (20 μg protein ml^{-1}) for 1 h. After three successive washes in TBST the membrane was incubated in TBST containing 1% milk and rabbit anti-chicken IgY peroxydase conjugates (Sigma, France) at a final dilution of 1:4000 for 1 h. Three washes were performed in TBST and the antigen was detected using the ECl system as previously described. The limit of detection was 200 ng of pure NDP kinase A (data not shown). The autoradiographs were scanned and the results expressed as positive if the scan could detect a band corresponding to a molecular weight of 18 kDa, and negative in the other cases. The pure NDP kinase (5 μg) loaded on the gel was used as an internal standard to avoid variations due to different exposures from one gel to another.

Statistical analysis

The association of Nme1 expression with other discrete clinicobiological variables was assessed by chi-square analysis. The relationships between Nme1 expression and cathepsin D, pS2 and c-erbB-2 were analysed as continuous variables using the Student's t-test. Disease-related death for specific survival and metastasis for metastasis-free survival were scored as events with censoring of other patients at the time of last follow-up. Survival curves were calculated by the method of Kaplan and Meier. Univariate analyses were performed with the log-rank test.

Results

Antibody specificity

The aim of this work was to obtain an antibody able to react specifically with Nme1 without any cross-reaction with the Nme2 form. Because of the high homology of the amino acid sequence (more than 88%) that exists between these two

proteins, we decided to immunise the animals with a synthetic peptide whose sequence is localised in a region of weak homology. This 16 amino acid peptide of Nme1 is located from glycine 37 to tyrosine 53 and represents less than 50% of sequence homology with the same region of Nme2.

We first tested the specificity of this antibody by Western blot analysis. As shown in Figure 1, Nme1 antibody was able to detect pure NDP kinase A whereas it did not react with pure NDP kinase B. Control IgY did not reveal any signal (data not shown).

Expression of Nme1 in normal and tumour tissues

The expression of Nme1 in cytosolic extracts from normal breast tissues and tumours was then analysed. The Nme1 antibody revealed a band corresponding to the molecular weight of NDP kinase A (18 kDa) (Figure 2) in breast tumours. This band was not present when the antibody was first incubated with 10 μg of peptide coupled to haemocyanin (Figure 2). No 18 kDa form of Nme1 could be detected in normal breast tissue (data not shown). We then analysed the expression of Nme1 in 59 human breast tumours by scanning the 18 kDa band on the Western blot. Our results are presented as 'Nme1 +' if the scan could detect a 18 kDa band and 'Nme1 −' in the other cases.

Relationship between Nme1 expression and other prognostic factors

We analysed the relationships that might exist between Nme1 expression and the other prognostic factors listed in Table I. No significant difference was detected between the expression of Nme1 and menopausal status, nodal invasion or tumour size (Table I). Concerning the histological grade, 74% of well-differentiated tumours (grades 1 and 2) expressed Nme1 but the relationship was not significant ($P = 7.2\%$) (Table I). A significant difference was noted according to oestrogen receptor status (ER): around 71% of ER-negative tumours did not express Nme1 whereas 81% of ER-positive tumours expressed Nme1 ($P < 0.001$). Progesterone receptor (PR)-positive tumours preferentially expressed Nme1 (74%) compared with PR-negative ones (50%) but the relationship was not significant (Table I). A possible relationship between Nme1 and tumour hormone dependence was tested by analysing the pS2 protein expression level in Nme1 + tumours vs Nme1 − (Table II). No significant difference could be observed in the pS2 protein level between Nme1 + tumours and Nme1 − tumours. Similar results were obtained when comparing Nme1 expression with the amount of cathepsin D and with erbB-2 expression: 17% of tumours
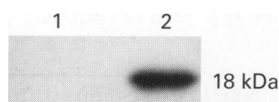
showed an overexpression of the ErbB-2 protein level (higher than 30 units). However, no significant difference in ErbB-2 expression level was observed between Nme1 − and Nme1 + tumours (Table II).

Relationship between Nme1 status, disease-free survival and overall survival

Of the 59 patients entering this study, 14 have developed metastasis during the 50 months following surgery, and 12 died from cancer. Median follow-up was 42 months. Univariate analysis did not show any relationship between metastasis-free survival or specific survival and histological grade, lymph node invasion, hormone receptors, cathepsin D or ErbB-2. It confirmed the predictive value of tumour size ($P < 0.01$) for specific survival and revealed that Nme1 expression was significantly associated with longer metastasis-free survival ($P < 0.001$) (Figure 3) and to overall survival ($P < 0.001$) (Figure 4).

Discussion

The results published by Steeg (1988) or Henessy (1991) suggested that *nme1* gene expression measured by mRNA analysis is related to the metastasis potential of human breast cancer. To analyse the potentialities of the tumour metastasis

Figure 1 Analysis of the hen antibody specificity: the Western blot analysis was performed on 4 μg of pure NDP Kinase B (lane 1) and 4 μg of pure NDP Kinase A (lane 2) as described in Materials and methods.
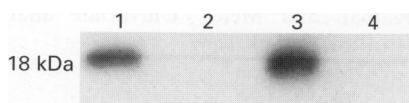

Figure 2 Nme1 expression in tumour tissues. The analysis was performed as described in Materials and methods, using 60 μg of cytosol from tumour tissues expressing Nme1 (lane 1) or not (lane 2) and pure Nme1 protein (lane 3). Preincubation of the antibody with 10 μg of antigen before Western blot analysis is shown in lane 4.

Table I Relationship between Nme1 expression and prognostic factors as discrete variables

Tumour and patient characteristics	Nme1 −	Nme1 +	P
Nodal status			
Negative	7	13	NS
Positive	13	26	
Tumour grade			
1 or 2	9	27	NS
3	11	12	
Tumour size			
<2 cm	4	13	NS
>2 cm	15	25	
Menopausal status			
Premenopausal	3	18	NS
Post-menopausal	9	23	
Oestrogen receptor status			
Negative	12	5	<0.001
Positive	8	34	
Progesterone receptor status			
Negative	10	10	NS
Positive	10	29	

Statistical analysis was performed as described in Materials and methods. NS, non-significant relationship.

Table II Relationship between Nme1 expression and prognostic factors as continuous variable

Tumour marker	Nme1 −	Nme1 +	P
Cathepsin D level			
Mean	48.2	42.2	
s.d.	36.6	22.7	NS
n	20	38	
pS2 level			
Mean	11.7	15.0	
s.d.	17.2	18.7	NS
n	20	38	
ErbB-2 level			
Mean	17.9	19.4	
s.d.	23.3	26.9	NS
n	15	27	

Statistical analysis was performed as described in Materials and methods. NS, non-significant relationship.
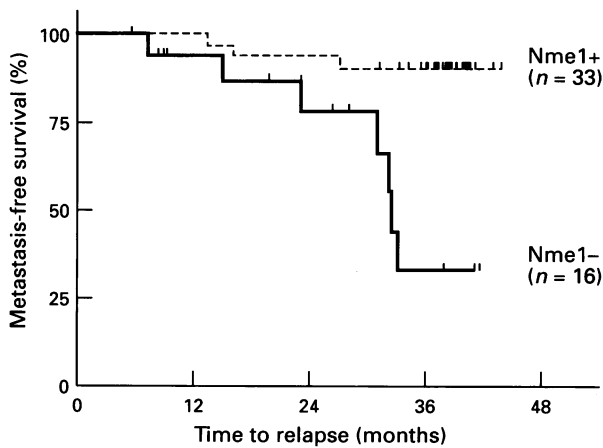
Figure 3 Metastasis-free survival curve according to Nme1 status: Nme1−, no band detectable at 18 kDa; Nme1+, a band detected by scan densitometer. The curve was calculated according to the Kaplan−Meier method and statistical analysis was performed as described in Materials and methods.
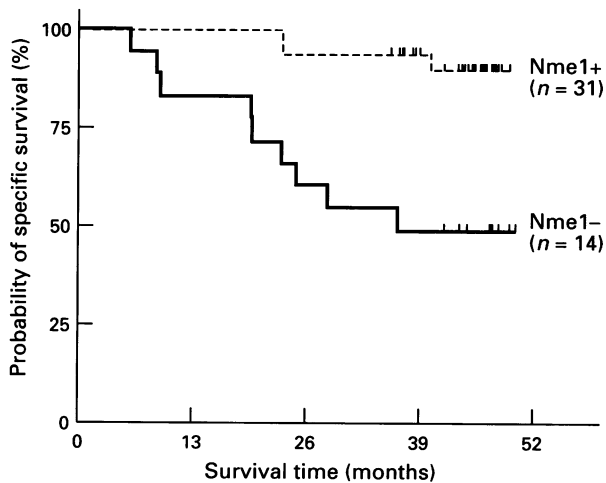

Figure 4 Specific survival curves according to Nme1 status: Nme1−, no band detectable at 18 kDa; Nme1+, a band detected by scan densitometer. The curve was calculated according to the Kaplan−Meier method and statistical analysis was performed as described in Materials and methods.

gene to predict metastasis outcome we raised an antibody against an Nme1 protein. Instead of immunising animals with the whole Nme1 protein a 16 amino acid peptide, derived from a region of the protein that represents a weak sequence homology, was injected into hens. The immunisation produced an antibody that specifically reacted with Nme1 without any cross-reaction with the Nme2 form. This highly specific tool was then used to analyse Nme1 protein expression in human breast tumours by Western blotting.

In our study, Nme1 was not detected in any of the four normal breast tissues tested. Immunohistochemical studies of Nme1 have given conflicting data concerning the staining of normal cells: in the study by Hiriyama *et al.* (1991) cells of normal acini and glands in the breast tissue generally could be weakly stained or not stained at all; Sastre-Gareau (1992), using a polyclonal anti-NDP kinase A, observed no or moderate staining of normal lobules and ducts; Simpson *et al.* (1994) reported that the percentage of Nm23 reactivity of lobular or ductal carcinoma *in situ* was greater than that of adjacent normal tissue. Other workers (Barnes *et al.*, 1991; Tokunaga *et al.*, 1993; Royds *et al.*, 1993), using different polyclonal antibodies, detected staining in normal breast. All the antibodies used in these studies recognised

both Nme1 and Nme2 and so detected the expression of both proteins in normal breast. That our antibody does not cross-react with Nme2 would explain the lower expression seen in our study; the level of Nme1 expression in normal breast cytosol is probably lower than the sensitivity limit of our hen antibody.

Our work analysed the possible relationships between Nme1 expression and other prognostic factors. No significant relationship was demonstrated either with age, menopausal status, tumour size, or lymph node status. Even if 74% of well-differentiated tumours expressed Nme1, no significant relationship could be detected between histological grade and Nme1 expression ($P = 7\%$). Studies by Henessy *et al.* (1991), Bevilacqua *et al.* (1989) and Royds *et al.* (1993) related Nme1 negative expression to histological grade and to worsening invasive ductal carcinoma grade. It could be supposed that by using a larger population than examined in our study, we would have revealed a link between Scarff − Bloom-Richardson (SBR) grade and Nme1 expression. On the other hand, a positive relationship between Nme1 expression and hormonal receptors has been shown in our study but this relationship was only significant for the oestrogen receptor ($P < 0.001$). Several workers (Adami *et al.*, 1985; Reiner, 1990) have related the amount of oestrogen receptor to the differentiation status of the tumour. Our data also suggest that *nme1* could be related to differentiation state of the tumour. This is in agreement with the recent observations of Howlett *et al.* (1994), who showed that *nme1*-transfected cell lines in culture with a reconstituted basement membrane presented characteristics of breast differentiation. Even if these results do not imply that the *nme1* gene universally controls breast differentiation, it may be one important gene in the process of differentiation.

Like the progesterone receptor, the expression of pS2 protein is oestrogen dependent (Brown *et al.*, 1984) and is predictive of a favourable response to endocrine therapy in human breast cancer (Henry *et al.*, 1991). In our work, no significant relationship was observed between Nme1 expression and the pS2 protein level. These data and the absence of a link between Nme1 expression and progesterone status would indicate that Nme1 is not related to hormone dependent tumour status.

The c-*erb*B-2 oncogene, which encodes for a transmembrane glycoprotein with high homology with epidermal growth factor receptor, is amplified in 15−30% of breast cancer. This overexpression has been found to be related to an increase in the proliferative activity of the tumour and by some authors to a shorter survival (Wright *et al.*, 1989; Tandon *et al.*, 1989; Rilke *et al.*, 1991). More recently, an overexpression of c-*erb*B-2 has been associated to the response to chemotherapy (Muss *et al.*, 1994). A significant relationship between the expression of c-*erb*B-2 and the mRNA level of *nme1* has been shown in ovarian carcinomas (Mandai *et al.*, 1994); this relationship was also observed by immunohistochemical staining of sections for *nme* products and c-*erb*B-2. On the contrary, Slamon (1991) reported that human breast and ovarian carcinoma cell lines transfected with HER2/neu exhibited reduced *nme* mRNA levels. In our population, an overexpression of c-*erb*B-2 was observed in 17% of tumours but no significant relationship between c-*erb*B-2 and Nme1 expression could be demonstrated. Furthermore, Nme1 expression was independent of another metastasis predictive factor, cathepsin D levels, suggesting that the metastasis process involving Nme1 protein could be different from that of cathepsin D.

We have described here highly significant relationships between Nme1 expression and metastasis-free survival ($P < 0.001$) and specific survival ($P < 0.001$). These results obtained at the level of protein expression using our antibody are in agreement with several studies analysing *nme1* mRNA levels in breast cancer (Henessy *et al.*, 1991) and in other locations (Arai *et al.*, 1993). Although this approach needs to be confirmed by a larger study, it underlines the interesting role of specifically detecting cytosolic *nme1* for the prediction of metastasis outcome in breast cancer.

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