

## ORIGINAL ARTICLE

## Increased nm23 immunoreactivity is associated with selective inhibition of systemic tumour cell dissemination

A N J Graham, P Maxwell, K Mulholland, A H Patterson, N Anderson, K G McManus, H Bharucha, J A McGuigan

*J Clin Pathol* 2002;**55**:184–190

**Aims:** In vitro transfection experiments show that the nm23 gene suppresses metastasis, although the evidence from clinical studies is contradictory. The purpose of this study was to investigate whether nm23 selectively influences systemic, pleural, and lymphatic metastasis in non-small cell lung cancer (NSCLC).

**Methods:** Forty two patients undergoing resection of NSCLC and lymph node sampling were enrolled prospectively. In each case, a bone marrow aspirate, pleural lavage, and lymph nodes were assessed using immunohistochemistry for epithelial antigens and morphology. The intensity of nm23-H1 immunoreactivity of the primary tumour was compared with the internal control of normal bronchial epithelium in 32 cases where available. The microvessel count (MVC) of each tumour was determined using immunohistochemistry for the endothelial cell marker CD34.

**Results:** Tumour cell dissemination was detected in the bone marrow in 18 patients, in the pleura in seven, and in the lymph nodes in 21. Increased immunoreactivity for nm23 was found in the primary tumour in six patients, with none having tumour cells in the bone marrow, compared with 12 of 26 patients who showed nm23 immunoreactivity equal to or less than the control (Fisher's exact test:  $p = 0.043$ ). This effect was confirmed to be independent of the MVC on multivariate analysis. There was no significant difference in the incidence of pleural or lymphatic tumour cell dissemination between the two groups.

**Conclusions:** nm23 appears to be a suppresser of systemic, but not lymphatic, metastasis in primary NSCLC.

See end of article for authors' affiliations

Correspondence to:  
Mr ANJ Graham, Royal Victoria Hospital, Grosvenor Road, Belfast BT12 6BA, Northern Ireland;  
[alastair.graham@royalhospitals.ni.nhs.uk](mailto:alastair.graham@royalhospitals.ni.nhs.uk)

Accepted for publication 31 July 2001

Somatic allelic deletion of the nm23 gene, localised to chromosome 17, has been seen in human breast, renal, colorectal, and lung carcinomas.<sup>1</sup> There are two human variants, nm23-H1 and nm23-H2, encoding 17 kDa proteins that are 90% identical<sup>2</sup>—nucleoside diphosphate kinases A and B.<sup>3</sup> The nm23 gene was first associated with metastasis in a study of murine K-1735 melanoma cell lines of high and low metastatic potential.<sup>4</sup> Lower expression of nm23 mRNA<sup>4</sup> and protein<sup>5</sup> was subsequently found in highly metastatic rat mammary carcinomas. When highly metastatic murine K-1735 TK melanoma cells were transfected with murine nm23-l cDNA the number of metastases was reduced by 58–96%, compared with the control transfected with empty vector,<sup>6</sup> and these findings have been confirmed in subsequent studies.<sup>7–8</sup> However, clinical studies in human cancers have produced conflicting results. Low amounts of nm23 RNA have been associated with metastasis in patients with breast cancer,<sup>9–10</sup> malignant melanoma,<sup>11</sup> and gastric cancer.<sup>12</sup> In non-small cell lung cancer (NSCLC), increased nm23 RNA expression tended to correlate with higher tumour stage and number of lymph node metastases and, in cases of squamous carcinoma, poorly differentiated tumours contained significantly more nm23 RNA.<sup>13</sup> Immunohistochemical studies in NSCLC have also given contrasting findings, with only three<sup>14–16</sup> of nine reported studies providing evidence to support the hypothesis that nm23 is a suppresser of metastasis. Expression of nm23 was unrelated to tumour stage or survival in four studies,<sup>17–20</sup> and in two high expression was associated with increased T stage and worse prognosis.<sup>21–22</sup>

“Low amounts of nm23 RNA have been associated with metastasis in patients with breast cancer, malignant melanoma, and gastric cancer”

These conflicting findings may result from differences in study design and the endpoints chosen. Although tumour deposits in lymph nodes were taken as the only endpoint for metastasis in most of these studies, there may be differences in the populations of tumour cells forming lymphatic and systemic metastases. Systemic metastasis is the end result of several steps, including cells entering the circulation, arrest at the distant vascular bed, extravasation into the target organ parenchyma, and proliferation as a secondary colony,<sup>23</sup> whereas the entry of tumour cells into the lymphatic system may depend on different mechanisms. Because each step may have different regulatory mechanisms, it is unlikely that a single factor in the primary tumour could predict established metastasis.

Dissemination of intact tumour cells to the three systems in which metastasis may develop in NSCLC may be detected using immunohistochemical localisation of epithelial antigens. Using this model, which focuses on an isolated but essential step in the metastatic cascade, we attempted to clarify the association between nm23 and metastasis in NSCLC. Because there is evidence that the microvessel count (MVC) determined using immunohistochemistry for endothelial cell antigens is a predictor of systemic metastasis<sup>24–27</sup> in NSCLC, the MVC of the tumours was determined to investigate whether any association between nm23 protein expression and tumour cell dissemination was independent of the degree of angiogenesis in the primary tumour.

**Abbreviations:** APES, aminopropyltriethoxysilane; CT, computed tomography; MVC, microvessel count; NDPK, nucleoside diphosphate kinase; NSCLC, non-small cell lung cancer; PBS, phosphate buffered saline

## MATERIALS AND METHODS

### Patients

Subjects were recruited from those scheduled to undergo thoracotomy in the Northern Ireland Regional Thoracic Surgery Unit for resection of histologically confirmed NSCLC. There were 42 patients enrolled, 10 of whom were women, with a mean age of 65.6 years (SD, 8.8). Ethical approval for the study was obtained from the Queen's University of Belfast medical ethics committee and written informed consent was obtained in all cases. Those with a previous history of malignant disease who had not had curative treatment were excluded, as were those who had a history of preoperative administration of chemotherapy or radiotherapy. To be included in our study, each patient had to have complete resection of the primary tumour and lymph node sampling, with a minimum of one N1 and two N2 node stations.

### Clinical staging protocol

All patients had undergone clinical staging by physical examination, measurement of serum alkaline phosphatase and transaminases, bronchoscopy, and contrast enhanced computed tomography (CT) scanning of the chest and upper abdomen. Invasive staging by mediastinoscopy or mediastinotomy was indicated if the CT scan showed mediastinal lymph nodes with a short axis diameter greater than 1.0 cm. This was performed before thoracotomy in three patients. Radioisotope bone scans were performed and were negative in three patients with symptoms suggestive of bone metastases.

### Pathological staging protocol

The pathological stage of the disease was classified according to the TNM classification and current stage grouping. The histological features of all the resection specimens were reviewed jointly by the same two pathologists to exclude interobserver differences in interpretation. In each case cell type, differentiation, pleural involvement, lymphovascular invasion, bronchial origin, adequacy of resection, and sections of all lymph nodes were reviewed. Squamous carcinomas were designated grade 1 if there was evidence of keratin formation, grade 2 if there was no keratin formation, and grade 3 if there was necrosis. Adenocarcinomas were classed as grade 1 if there were well formed glands, grade 2 if there were poorly formed glands, and grade 3 if glandular structures were absent and there was sheeting of cells.

### Retrieval of tissue samples

#### Bone marrow

The bone marrow aspiration was performed under general anaesthesia before the thoracotomy was commenced. In all cases, the site of aspiration was the posterior iliac crest on the same side of the body as the pulmonary lesion. Two direct smears were immediately made from the first 0.5 ml aspirated. These were air dried and stored at room temperature for subsequent staining with May-Grunwald-Giemsa. A further 10 ml of bone marrow was aspirated into a syringe containing 2500 IU heparin and added to 10 ml phosphate buffered saline (PBS) for the preparation of cytopspins.

#### Pleural lavage

Immediately after the parietal pleura was opened the surface of the lung was irrigated with 50 ml sterile normal saline. The washings were retrieved using suction attached to a trap specimen container and transferred to 50 ml PBS containing 2500 IU heparin.

#### Primary tumour

The primary tumour was resected by the operation deemed most appropriate by the operating surgeon. In 29 patients this was lobectomy, in nine pneumonectomy, in three anatomical segmentectomy, and in one wedge resection. The resected

specimen was transported in a 10% solution of formalin to the pathology department for histological assessment.

### Lymph nodes

Lymph node sampling was performed at thoracotomy with at least one bronchopulmonary and two mediastinal levels sampled for each patient. The median number of levels assessed was four (range, three to six). All nodes were labelled separately and submitted intact by the surgeon. They were transported in a 10% solution of formaldehyde to the pathology department for histological assessment, where they were sectioned once before haematoxylin and eosin staining.

### Preparation of bone marrow and lavage specimen cytopspins

The two methods used for preparing cytopspins were ammonium chloride red blood cell lysis and density gradient centrifugation through Ficoll (Pharmacia, Sweden) and are detailed below. Experiments were performed to determine the optimum technique and both were found to be suitable for preparing cytopspins from bone marrow aspirates (data not shown). However, it was determined that at least  $3.5 \times 10^6$  nucleated cells harvested by lysis had to be examined to give an equivalent result to the assessment of  $2 \times 10^6$  nucleated cells prepared by density gradient centrifugation.

#### Ammonium chloride lysis

The 20 ml sample of bone marrow aspirate in PBS was centrifuged at  $100 \times g$  for five minutes and the cells in the resulting pellet were resuspended in 5 ml of the supernatant. Next, 20 ml of 8% ammonium chloride was added and mixed by inversion until the sample became translucent. The ammonium chloride was removed by centrifugation at  $100 \times g$  for five minutes and the cells in the deposit were washed twice, then resuspended in 5 ml fresh PBS.

#### Density gradient centrifugation

The 20 ml sample was layered carefully on to Ficoll and centrifuged at  $400 \times g$  for 30 minutes in a temperature controlled centrifuge at 20°C (MSE Mistral 3000i). The cells from the buffy coat at the interface were retrieved, resuspended in PBS, and washed twice by centrifugation at  $100 \times g$  for five minutes, then resuspended in 5 ml fresh PBS.

### Cytopspins

The concentration of nucleated cells in each resuspended sample was determined by an automated cell count on the SE9500 (Sysmex, Milton Keynes, UK). This was then adjusted by dilution to  $1.34 \times 10^6$ /litre. Cytopspins were made, each from 150  $\mu$ l ( $2 \times 10^6$  nucleated cells), on aminopropyltriethoxysilane (APES) coated slides and immediately fixed and stored in 95% alcohol at room temperature.

### Immunohistochemistry

#### Bone marrow and pleural lavage cytopspins

Immunohistochemical staining of the cytopspins was performed with the anticytokeratin antibodies AE1/AE3 (Dako, Ely, UK), at a concentration of 0.8  $\mu$ g/ml, and CAM 5.2 (Becton Dickinson, Oxford, UK) at a concentration of 0.5  $\mu$ g/ml. Briefly, after endogenous peroxidase activity was blocked by immersion for 10 minutes in 3% alcoholic hydrogen peroxide, the cytopspins were pretreated with a 1/20 dilution of swine serum for 15 minutes and the primary antibody applied for 30 minutes at room temperature. One cytopspin from each sample was used as a negative control and incubated with mouse IgG1 in place of the primary antibody at an equivalent concentration. The immunological staining was completed by a standard strep-avidin-biotin complex method (Dako).

**Table 1** The incidence of bone marrow and pleural tumour cell dissemination in relation to the histological features of the resected tumour

	Total	Positive bone marrows	p Value	Positive pleural lavages	p Value
Squamous carcinoma	29	10	0.101	4	0.369
Adenocarcinoma	13	8		3	
Grade 1	7	4	0.645	1	0.917
Grade 2	21	9		4	
Grade 3	14	5		2	
>1mm between tumour and pleura	28	11	0.685	5	0.874
≤1mm between tumour and pleura	9	5		1	
Visceral pleura invaded	5	2		1	
No vascular invasion	30	13	0.805	4	0.312
Vascular invasion identified	12	5		3	
No lymphatic invasion	32	12	0.336	5	0.534
Lymphatic invasion identified	10	6		2	
No bronchial origin	24	9	0.418	4	0.665
Bronchial origin identified	18	9		3	

Results of assessment of bone marrow aspirates and pleural lavages for tumour cell dissemination in the 42 patients enrolled; p values were calculated using the  $\chi^2$  test.

### Lymph nodes

Representative lymph node stations with no metastases identified on histological review were immunostained with AE1/AE3 to aid identification of disseminated tumour cells. For each tumour the representative N1 node was the relevant lobar node. For upper lobe tumours the tracheobronchial on the right or aortopulmonary window node on the left were selected for the N2 nodes; for lower and middle lobe tumours the subcarinal nodes were stained.

### Primary tumour and lymph nodes

Sections from each primary tumour and, in 14 of the cases, of a lymph node metastasis were cut at 4  $\mu$ m on to APES (Sigma, Poole, Dorset, UK) coated slides. Immunohistochemistry for nm23-H1 was performed with mouse monoclonal m23 clone NM301 (Ab-1; Oncogene Research Products, Cambridge, Massachusetts, USA) at 5  $\mu$ g/ml, with overnight incubation. Immunostaining with anti-CD34 mouse monoclonal antibody (Serotec, Kidlington, Oxford, UK) at 10  $\mu$ g/ml for 30 minutes was performed. Immunolocalisation was visualised using a standard strep-avidin-biotin complex STABC method (Dako). Diaminobenzidine (Dako) was used as the chromogen and sections were counterstained in Harris's haematoxylin.

## ASSESSMENT OF RESULTS

### Tumour cell dissemination

All slides were screened by one observer and the findings verified by a specialist cytopathologist, or in the case of lymph nodes, by a histopathologist. The morphological characteristics of immunopositive cells identified on cytopins were assessed. Cells were only confirmed to be malignant when all of the following were present:

- (1) size larger than mature blood cells
- (2) nuclear pleomorphism
- (3) increased nuclear to cytoplasm ratio.

Immunopositive cells demonstrating these features were considered to be true positives, those without were considered to be false positives.

### nm23-H1 immunoreactivity

The intensity of nm23-H1 immunoreactivity in the tumour was assessed by two independent, experienced observers and categorised as less than, equal to, or greater than that of normal bronchial epithelium where this could be identified on the same slide. Cases in which the results differed were reviewed jointly by the two observers and an agreement was reached.

### Microvessel count

The area of greatest vascularity was determined by low power microscopy and the number of discrete discontinuous endothelial cell clusters within a fixed field size of 0.41 mm<sup>2</sup> counted using the Kontron Interactive Image Analysis System (Kontron Elektronik, Munich, Germany), comprising the Videoplan interactive software package and a 3CCP colour camera (Sony, Weybridge, Surrey, UK). An Olympus BH1 microscope (Olympus, London, UK) was used at a magnification of  $\times 100$ . The hotspot was identified and then viewed on a visual display unit. As each microvessel was counted a cursor was used to insert a red cross on the screen over each vessel, to prevent it being counted more than once, and the numbers of red crosses were counted automatically. The interobserver and intra-observer variability of the methodology for MVC was tested and found to be reproducible (data not shown). All slides were assessed by two independent observers who were blinded to the other's findings, and to the stage of the tumour or outcome of the patient. The mean of their two results was used to determine the MVC used in the statistical analysis.

### Statistical analysis

Statistical analysis was performed using Statistica version 5.1 (StatSoft Inc, Tulsa, Oklahoma, USA). Categorical variables were compared using the  $\chi^2$  test unless otherwise stated. Continuous variables were compared using the Student's *t* test for two independent variables or one way ANOVA and the Student-Newman-Keuls test for comparisons of more than two means.

Spearman's rank method was used for correlation matrices. Multivariate analysis was carried out by stepwise discriminant analysis. Significance was set at  $p < 0.05$ , and the *p* values were not adjusted to take account of multiple comparisons.

## RESULTS

### Tumour cell dissemination in bone marrow aspirates

Malignant cells were identified in the bone marrow aspirates of 18 patients, nine on assessment of cytopins and nine on smears. Immunopositive cells were identified on the cytopins of 22 cases; however, in only nine cases were malignant cells identified after morphological assessment. Tumour cell dissemination was more frequently detected in the bone marrow aspirates from patients with adenocarcinomas than those with squamous carcinomas, although this was not significant ( $p = 0.101$ ; table 1). There was no significant association with

**Table 2** The incidence of bone marrow and pleural tumour cell dissemination in relation to the post surgical stage of the tumour

	Total assessed	Positive bone marrow aspirates	p Value	Positive pleural lavages	p Value
T1	8	5	0.172†	2	0.414†
T2	33	12		5	
T3	1	1			
N0	25	12	0.711*	2	0.109*
N1	6	2		1	
N2	11	4		4	
Stage 1	24	11	0.871*	2	0.116*
Stage 2	7	3		1	
Stage 3	11	4		4	

Results of assessment of bone marrow aspirates and pleural lavages for tumour cell dissemination in the 42 patients enrolled.

\* $\chi^2$  test; †T1 v T2.

the grade of tumour or the presence of lymphovascular invasion (table 1). There was no association with T, N, or combined stage (table 2).

### Tumour cell dissemination in pleural lavages

Malignant cells were confirmed in seven pleural lavages. There were no significant differences in the characteristics of the resected primary tumour in the patients with and without malignant cells in pleural lavages (table 1). Fine needle aspiration of the tumour for diagnostic purposes was performed before thoracotomy in 15 patients: one had positive pleural lavages at thoracotomy compared with six of the 27 who had not undergone this procedure ( $p = 0.198$ ). Malignant cells were more commonly found in pleural lavages of patients with increasing N category (table 2), although this was not significant ( $p = 0.109$ ). Overall, tumour cell dissemination was present in all three systems in one patient, in two systems in 13, and in one system in 17. Only 11 had no tumour cell dissemination detected.

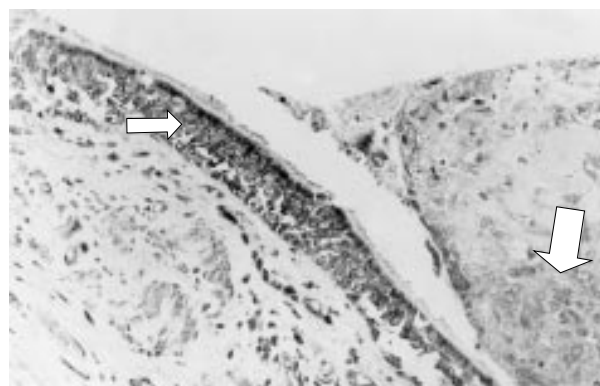
### Tumour cell dissemination in lymph nodes

Of 31 patients classified as N0 after histological review, five were determined to have malignant cells present in lymph nodes after immunohistochemical staining and assessment. The involved level was N1 in two patients and N2 in four (one patient had involvement of both levels). There was no significant difference in the incidence in the groups with and without lymphatic invasion observed on the histological review of the resected tumour. Adequate lymph node sampling had been performed on all 42 patients enrolled, with 17 being found to have tumour cell dissemination in the lymph nodes. After the immunohistochemically aided assessment of negative lymph nodes in 31 patients, this number increased to 22.

### nm23-H1 immunoreactivity

In 32 of the 42 cases assessed, normal bronchial epithelium could be identified adjacent to the primary tumour and in all except one this exhibited positive immunoreactivity for nm23. There was polarity of immunoreactivity in the normal bronchial epithelium in all cases, with the luminal aspect of the cell showing stronger staining than the basal part (fig 1). All primary tumours showed immunoreactivity of varying levels for nm23. In all cases, the polarity of immunoreactivity exhibited in normal bronchial epithelium was lost in the tumour cells. The staining of tumour cells was almost exclusively cytoplasmic, with two cases demonstrating additional small areas of apparent membrane staining.

In the 32 cases in which normal bronchial epithelium could be identified on the same section as the primary tumour, it was used as an internal control to score the intensity of nm23-H1 immunoreactivity of the tumour. The intensity of cytoplasmic nm23-H1 immunoreactivity of the primary



**Figure 1** Immunoreactivity of normal bronchial epithelium for nm23-H1 showing polarity of staining (thin arrow). Adjacent tumour shows less intense immunoreactivity without polarity (thick arrow).

tumour was less than, equal to, and greater than that of the normal bronchial epithelium in 12, 14, and six cases, respectively (table 3).

### Tumour cell dissemination and nm23-H1 immunoreactivity

The incidence of tumour cell dissemination in the three systems was cross tabulated with the nm23-H1 immunointensity of the primary tumours. Of the six cases with nm23-H1 tumour immunoreactivity greater than bronchial epithelium, none had bone marrow tumour cell dissemination, compared with 12 of 26 with tumour immunoreactivity less than or equal to the internal control ( $p = 0.043$ ; table 4). When the results for bone marrow and pleural tumour cell dissemination were combined the association was stronger. Of the six where the intensity of nm23-H1 immunoreactivity in the tumour was greater than that in the bronchial epithelium, none had bone marrow or pleural tumour cell dissemination, compared with 14 of the 26 where it was less than or equal to the internal control ( $p = 0.009$ ; table 4). When those with lymphatic metastasis diagnosed on histological review were excluded, none of the four cases where the tumour nm23-H1 immunoreactivity was greater than that in the bronchial epithelium had bone marrow or pleural tumour cell dissemination, compared with seven of the 13 where it was less than or equal to the control ( $p = 0.09$ ).

### Microvessel count

All cases demonstrated immunopositivity of blood vessels and endothelial cells. There was a significant correlation in the MVC determined by the two independent observers in the 32 tumours in which nm23-H1 immunopositivity could be

**Table 3** Tumour immunoreactivity (TI) for nm23-H1 (compared with normal bronchial epithelium (NBE)) in relation to the histological features and stage of the resected tumour

	n	TI<NBE	TI=NBE	TI>NBE
Squamous carcinoma	21	8	7	6
Adenocarcinoma	11	4	7	0
Grade 1	6	4	2	0
Grade 2	16	5	7	4
Grade 3	10	3	5	2
T1	6	4	2	0
T2	26	8	12	6
N0	17	7	6	4
N1	5	0	4	1
N2	10	5	4	1
Stage 1	17	7	6	4
Stage 2	5	0	4	1
Stage 3	10	5	4	1

In 32 of the 42 cases assessed by immunohistochemistry, normal bronchial epithelium could be identified on the same section. The intensity of immunoreactivity in the tumours in these 32 cases was compared with the respective normal bronchial epithelium.

**Table 4** Tumour immunoreactivity (TI) for nm23-H1 (compared with normal bronchial epithelium (NBE)) correlated with tumour cell dissemination (TCD) in all cases in which normal epithelium was identified on the tissue section

	n	TI<NBE	TI=NBE	TI>NBE
Bone marrow TCD absent	20	6	8	6
Bone marrow TCD detected	12	6	6	0
Pleural TCD absent	26	8	12	6
Pleural TCD detected	6	4	2	0
Lymphatic TCD absent	14	7	4	3
Lymphatic TCD detected	18	5	10	3
TCD absent in all systems	9	2	4	3
TCD detected in at least 1 system	23	10	10	3
Bone marrow and pleural TCD absent	16	3	7	6
TCD detected in bone marrow and/or pleura	16	9	7	0

In 32 of the 42 cases assessed by immunohistochemistry, normal bronchial epithelium could be identified on the same section. The intensity of immunoreactivity in the tumours in these 32 cases was compared to the respective normal bronchial epithelium.

assessed ( $r = -0.750$ ;  $p < 0.001$ ). There was a significant correlation between the maximum diameter of the resected tumour and the MVC in adenocarcinomas ( $r = 0.542$ ;  $p = 0.037$ ), but not in squamous carcinomas ( $r = -0.019$ ;  $p = 0.917$ ). The mean MVC of adenocarcinomas was significantly higher than that of squamous carcinomas ( $p = 0.010$ ) (table 5). There was no significant difference in the mean MVC of the different T and N categories or tumour stage. There was no significant difference in the mean MVC of tumours that had tumour cell dissemination detected and those that did not.

#### MVC and nm23-H1 immunoreactivity

As described previously, tumours with stronger nm23-H1 immunoreactivity than the normal bronchial epithelium had a reduced incidence of systemic tumour cell dissemination. The mean MVC of these tumours was 42.4 (SD, 30.3), not significantly less than the mean of 66.6 (SD, 34.2) in those with immunoreactivity equal to or less than the internal control ( $p = 0.151$ ).

#### Multivariate analysis

Stepwise discriminant analysis was performed with the tumour cell dissemination detected in bone marrow aspirates and/or pleural lavages as the dependent variable. The independent variables were T category, N category, tumour type (squamous carcinoma or adenocarcinoma), MVC of primary tumour, and nm23-H1 immunoreactivity of the tumour compared with normal bronchial epithelium. Significant independent predictors of systemic tumour cell dissemination were nm23-H1 immunoreactivity less than or equal to

**Table 5** Mean microvessel counts (MVC) in the standard histological subgroups

	n	MVC	SD	p Value
Squamous carcinoma	29	54.7	27.5	0.010*
Adenocarcinoma	13	82.2	35.0	
Grade 1	7	86.1	31.6	0.090†
Grade 2	21	62.6	29.0	
Grade 3	14	53.2	34.1	
No vascular invasion	30	63.6	30.6	0.976*
Vascular invasion	12	63.9	38.3	
No lymphatic invasion	32	61.0	31.4	0.377*
Lymphatic invasion	10	71.6	35.9	

MVC was determined in the 42 primary tumours that were assessed for nm23-H1 immunoreactivity. The mean values/standard field (size 0.41mm<sup>2</sup>) are shown.

\*Student's *t* test; †One way ANOVA (grade 1 v 3;  $p = 0.045$ ; Student-Newman-Keuls test).

bronchial epithelium ( $p = 0.464$ ;  $p < 0.001$ ) and adenocarcinoma cell type ( $p = 0.392$ ;  $p = 0.024$ ).

#### DISCUSSION

In our study, nm23-H1 protein expression was found in all cases of normal bronchial epithelium and was used as an indicator of normal epithelial cell immunoreactivity. All primary tumours expressed nm23-H1, but without the polarity that was evident in the normal bronchial epithelium. When the nm23-H1 immunoreactivity of the tumours was compared with that of the normal bronchial epithelium, a significant

correlation was found with the incidence of tumour cell dissemination in bone marrow aspirates and/or pleural lavages. In the patients with stronger nm23-H1 expression in the tumour than the bronchial epithelium, there were no cases of tumour cell dissemination, whereas, in the patients with tumour nm23-H1 expression less than the bronchial epithelium, 75% had tumour cell dissemination detected. The negative correlation between nm23-H1 and bone marrow tumour cell dissemination was significant when patients with established lymphatic metastases were excluded.

"All primary tumours expressed nm23-H1, but without the polarity that was evident in the normal bronchial epithelium"

There is only one previously published study correlating expression of the nm23 gene product with tumour cell dissemination in NSCLC; however, this only included the assessment of the lymphatic mode of spread.<sup>16</sup> In contrast to our current study, it was found that nm23 expression was associated with reduced tumour cell dissemination to lymph nodes. However, the methodological differences preclude direct comparison between the two studies. For example, the earlier study used a different antibody that detected both nm23-H1 and nm23-H2,<sup>16</sup> whereas we used an antibody that only detected nm23H-1, because this has been more closely associated with antimetastatic behaviour.<sup>7, 28</sup> In addition, the use of an internal control—normal bronchial epithelium—provides an objective semiquantitative means of assessing tumour nm23 immunoreactivity. Moreover, our study is the only one to correlate nm23 protein expression with tumour cell dissemination and identify the three potential sites of metastasis.

Although cases with strong expression of nm23-H1 had a lower MVC than those with tumour immunoreactivity equal to or less than the internal control, this difference was not significant. On multivariate analysis, the significant association between nm23-H1 immunoreactivity and reduced systemic tumour cell dissemination was confirmed to be independent of the degree of angiogenesis. This supports the findings of Heimann *et al*, who showed that nm23-H1 immunoreactivity and MVC were significant independent prognostic factors in patients with node negative breast cancers.<sup>29, 30</sup> They found that although a high MVC was a significant predictor of recurrence, those patients whose tumours had a high MVC and high immunoreactivity of nm23-H1 had a low rate of recurrence. Although it has been reported that there is a strong association between MVC and subsequent systemic metastasis,<sup>31</sup> two recent studies have not confirmed this<sup>32, 33</sup> and, therefore, a causative association between the degree of angiogenesis in primary NSCLC, as determined by the MVC, and metastasis has not yet been firmly established. This may be related to the different methods of data interpretation in the various studies.

The mechanism of action of nm23 is not clear. The main biochemical activity of the nm23 proteins appears to be an *in vitro*, non-specific nucleoside diphosphate kinase (NDPK) activity, which transfers the 5' phosphate from any nucleoside triphosphate to any nucleoside diphosphate via a high energy nm23 phosphohistidine intermediate.<sup>34</sup> However, NDPK activity fails to correlate with biological changes in differentiation and metastatic potential. Recent evidence has shown that nm23-H1 can transfer a phosphate from histidine to aspartate or glutamate residues on 43 kDa membrane proteins, and this correlates with the suppression of motility.<sup>34</sup> Because the nm23 protein has sequence homology over the entire translated region with a recently described developmentally regulated protein in *Drosophila*, mutations of which cause widespread aberrant differentiation,<sup>5</sup> it may be hypothesised that the nm23 gene product plays a role in cell to cell

### Take home messages

- In those patients with stronger nm23-H1 expression in the tumour than the bronchial epithelium, there were no cases of tumour cell dissemination, whereas 75% of the patients with tumour nm23-H1 expression less than the bronchial epithelium had tumour cell dissemination. This negative correlation between nm23-H1 and bone marrow tumour cell dissemination remained significant when patients with established lymphatic metastases were excluded
- Although cases with strong expression of nm23-H1 had a lower microvessel count than those with tumour immunoreactivity equal to or less than the internal control, this difference was not significant
- nm23 appears to suppress the dissemination of tumour cells from non-small cell lung cancer into the bloodstream, but not into the lymphatic system

interaction. This might be supported by our finding that in all cases there was polarity of immunolocalisation of normal bronchial epithelium that was lost in malignant cells, and in a small proportion there was apparent membrane staining. It may be hypothesised that systemic metastasis results from an active process of cell migration from the primary tumour, in which cell to cell interactions are important, whereas lymphatic metastasis results from increasing tissue pressure as a result of tumour growth causing malignant cells to be extruded into the lymphatic system. It has previously been found that the incidence of lymph node metastasis is higher in tumours originating close to the hilum of the lung,<sup>35</sup> where expansion of a tumour may cause higher pressure within the tissues than if it were sited peripherally in easily compressible pulmonary parenchyma.

The findings of our study support the hypothesis that nm23 may act as a metastasis suppressor gene affecting systemic and lymphatic dissemination of cells in differing ways. It appears to suppress the dissemination of tumour cells from NSCLC into the bloodstream, but not into the lymphatic system. Further research into the mechanism of this action may help elucidate the basic biology of lung cancer and may eventually lead to improvements in the management of patients suffering from this disease.

### ACKNOWLEDGEMENTS

This work was made possible by the award of a Royal Victoria Hospital Research Fellowship to AG, and we are grateful to the hospital for this. We are also grateful for grant aid from the Northern Ireland Thoracic Surgery Research Fund.

### Authors' affiliations

**A N J Graham, K G McManus, J A McGuigan**, Northern Ireland Regional Department of Thoracic Surgery, Royal Victoria Hospital, Grosvenor Road, Belfast BT12 6BA, Northern Ireland  
**P Maxwell, K Mulholland, A H Patterson, N Anderson**, Department of Pathology, Royal Victoria Hospital  
**H Bharucha**, Institute of Pathology, The Queen's University of Belfast, Belfast, BT12 6BL, Northern Ireland

### REFERENCES

- 1 Leone A, McBride OW, Weston A, *et al*. Somatic allelic deletion of nm23 in human cancer. *Cancer Res* 1991;**51**:2490-3.
- 2 de la Rosa A, Williams RL, Steeg PS. Nm23/nucleoside diphosphate kinase: toward a structural and biochemical understanding of its biological functions. *Bioessays* 1995;**17**:5362.
- 3 Gilles AM, Presecan E, Vonica A, *et al*. Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. *J Biol Chem* 1991;**266**:8784-9.
- 4 Steeg PS, Bevilacqua G, Kopper L, *et al*. Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst* 1988;**80**:200-4.
- 5 Rosengard AM, Krutzsch HC, Shearn A, *et al*. Reduced Nm23/Awd protein in tumour metastasis and aberrant *Drosophila* development. *Nature* 1989;**342**:177-80.

- 6 Leone A, Flatow U, King CR, *et al*. Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. *Cell* 1991;**65**:25–35.
- 7 Leone A, Flatow U, VanHoutte K, *et al*. Transfection of human nm23-H1 into the human MDA-MB-435 breast carcinoma cell line: effects on tumor metastatic potential, colonization and enzymatic activity. *Oncogene* 1993;**8**:2325–33.
- 8 Parhar RS, Shi Y, Zou M, *et al*. Effects of cytokine-mediated modulation of nm23 expression on the invasion and metastatic behavior of B16F10 melanoma cells. *Int J Cancer* 1995;**60**:204–10.
- 9 Bevilacqua G, Sobel ME, Liotta LA, *et al*. Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. *Cancer Res* 1989;**49**:5185–90.
- 10 Hennessy C, Henry JA, May FE, *et al*. Expression of the antimetastatic gene nm23 in human breast cancer: an association with good prognosis. *J Natl Cancer Inst* 1991;**83**:281–5.
- 11 Florenes VA, Aamdal S, Myklebost O, *et al*. Levels of nm23 messenger RNA in metastatic malignant melanomas: inverse correlation to disease progression. *Cancer Res* 1992;**52**:6088–91.
- 12 Nakayama H, Yasui W, Yokozaki H, *et al*. Reduced expression of nm23 is associated with metastasis of human gastric carcinomas. *Jpn J Cancer Res* 1993;**84**:184–90.
- 13 Engel M, Theisinger B, Selb T, *et al*. High levels of nm23-H1 and nm23-H2 messenger RNA in human squamous-cell lung carcinoma are associated with poor differentiation and advanced tumor stages. *Int J Cancer* 1993;**55**:375–9.
- 14 Lai WW, Wu MH, Yan JJ, *et al*. Immunohistochemical analysis of nm23-H1 in stage I non-small cell lung cancer: a useful marker in prediction of metastases. *Ann Thorac Surg* 1996;**62**:1500–4.
- 15 Kawakubo Y, Sato Y, Koh T, *et al*. Expression of nm23 protein in pulmonary adenocarcinomas: inverse correlation to tumor progression. *Lung Cancer* 1997;**17**:103–13.
- 16 Ohta Y, Nozawa H, Tanaka Y, *et al*. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. *J Thorac Cardiovasc Surg* 2000;**119**:804–13.
- 17 Higashiyama M, Doi O, Yokouchi H, *et al*. Immunohistochemical analysis of nm23 gene product/NDP kinase expression in pulmonary adenocarcinoma: lack of prognostic value. *Br J Cancer* 1992;**66**:533–6.
- 18 MacKinnon M, Kerr KM, King G, *et al*. p53, c-erbB-2 and nm23 expression have no prognostic significance in primary pulmonary adenocarcinoma. *Eur J Cardiothorac Surg* 1997;**11**:838–42.
- 19 Bosnar MH, Pavelic K, Hrascan R, *et al*. Loss of heterozygosity of the nm23-H1 gene in human renal cell carcinomas. *J Cancer Res Clin Oncol* 1997;**123**:485–8.
- 20 Tomita M, Ayabe T, Matsuzaki Y, *et al*. Immunohistochemical analysis of nm23-H1 gene product in node-positive lung cancer and lymph nodes. *Lung Cancer* 1999;**24**:11–16.
- 21 Ozeki Y, Takishima K, Mamiya G. Immunohistochemical analysis of nm23/NDP kinase expression in human lung adenocarcinoma: association with tumor progression in Clara cell type. *Jpn J Cancer Res* 1994;**85**:840–6.
- 22 Gazzeri S, Brambilla E, Negoescu A, *et al*. Overexpression of nucleoside diphosphate/kinase A/nm23-H1 protein in human lung tumors: association with tumor progression in squamous carcinoma. *Lab Invest* 1996;**74**:158–67.
- 23 Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991;**64**:327–36.
- 24 Fontanini G, Bigini D, Vignati S, *et al*. Microvessel count predicts metastatic disease and survival in non-small cell lung cancer. *J Pathol* 1995;**177**:57–63.
- 25 Fontanini G, Lucchi M, Vignati S, *et al*. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J Natl Cancer Inst* 1997;**89**:881–6.
- 26 Matsuyama K, Chiba Y, Sasaki M, *et al*. Tumor angiogenesis as a prognostic marker in operable non-small cell lung cancer. *Ann Thorac Surg* 1998;**65**:1405–9.
- 27 Yamazaki K, Abe S, Takekawa H, *et al*. Tumor angiogenesis in human lung adenocarcinoma. *Cancer* 1994;**74**:2245–50.
- 28 Caligo MA, Cipollini G, Flore L, *et al*. NM23 gene expression correlates with cell growth rate and S-phase. *Int J Cancer* 1995;**60**:837–42.
- 29 Heimann R, Ferguson DJ, Hellman S. The relationship between nm23, angiogenesis, and the metastatic proclivity of node-negative breast cancer. *Cancer Res* 1998;**58**:2766–71.
- 30 Heimann R, Lan F, McBride R, *et al*. Separating favorable from unfavorable prognostic markers in breast cancer: the role of E-cadherin. *Cancer Res* 2000;**60**:298–304.
- 31 Macchiarini P, Fontanini G, Hardin MJ, *et al*. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992;**340**:145–6.
- 32 Decaussin M, Sartelet H, Robert C, *et al*. Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival. *J Pathol* 1999;**188**:369–77.
- 33 Pastorino U, Andreola S, Tagliabue E, *et al*. Immunocytochemical markers in stage I lung cancer: relevance to prognosis. *J Clin Oncol* 1997;**15**:2858–65.
- 34 Wagner PD, Steeg PS, Vu ND. Two-component kinase-like activity of nm23 correlates with its motility-suppressing activity. *Proc Natl Acad Sci U S A* 1997;**94**:9000–5.
- 35 Graham AN, Chan KJ, Pastorino U, *et al*. Systematic nodal dissection in the intrathoracic staging of patients with non-small cell lung cancer. *J Thorac Cardiovasc Surg* 1999;**117**:246–51.

## ECHO

## Severity of meningococcal disease



Please visit the Journal of Clinical Pathology website [[www.jclinpath.com](http://www.jclinpath.com)] for link to this full article.

The number of bacteria in the blood determines the severity of meningococcal disease, predictably perhaps, but only recently shown in a study involving Alder Hey Children's Hospital, Liverpool, and the PHLS Meningococcal Reference Unit (MRU) in Manchester, UK.

Meningococcal disease can present as meningitis or septicaemia, or both; septicaemia carries a mortality of 6–75%. Its severity seems to hinge on patients' serum concentrations of certain cytokines, bacterial lipo-oligosaccharide (LOS) endotoxin, and bacterial capsular polysaccharide antigen. LOS and antigen concentrations may or may not be proportional to bacterial numbers.

The researchers used Taqman polymerase chain reaction (PCR) of a universal meningococcal capsular gene to measure bacterial numbers accurately in blood samples taken at admission and sequentially from children with probable/possible meningococcal disease. With one gene per cell, the number of genome copies measured per ml of blood equals bacterial load (viable and dead bacteria). Higher bacterial load at admission occurred with severe disease ( $\geq 8$  on Glasgow Meningococcal Septicaemia Prognostic Score) (median load  $8.4 \times 10^6$  severe versus  $1.1 \times 10^6$  mild disease), particularly in septicaemia patients ( $1.6 \times 10^7$  versus  $9.2 \times 10^5$ ;  $p < 0.001$ ). Loads were highest in two patients who died ( $p = 0.017$ ).

The method detected higher bacterial loads than other quantitative methods. Whether increased load and greater severity equates with serum bacterial antigen concentration or LOS endotoxin requires more work. The Taqman method, and speedier new PCR technology, the authors envisage, could in future be used to identify patients with the worst disease, who might then benefit from further treatment against cytokines and endotoxin.

▲ *Archives of Disease in Childhood* 2002;**86**:44–46.



## Increased nm23 immunoreactivity is associated with selective inhibition of systemic tumour cell dissemination

A N J Graham, P Maxwell, K Mulholland, et al.

*J Clin Pathol* 2002 55: 184-189

doi:

---

Updated information and services can be found at:

<http://jcp.bmj.com/content/55/3/184.full.html>

---

*These include:*

### References

This article cites 35 articles, 16 of which can be accessed free at:

<http://jcp.bmj.com/content/55/3/184.full.html#ref-list-1>

Article cited in:

<http://jcp.bmj.com/content/55/3/184.full.html#related-urls>

### Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

---

### Topic Collections

Articles on similar topics can be found in the following collections

[Immunology \(including allergy\)](#) (1279 articles)

[Lung cancer \(oncology\)](#) (76 articles)

[Lung cancer \(respiratory medicine\)](#) (76 articles)

---

### Notes

---

To request permissions go to:

<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:

<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:

<http://group.bmj.com/subscribe/>