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

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.

Original Article

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Abbreviations: APE5, 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 sheets 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 until the sample became translucent. The ammonium chloride was removed by centrifugation at 100 g for five minutes and the cells in the deposit were washed twice, then resuspended in 5 ml fresh PBS.

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 and added to 10 ml phosphate buffered saline (PBS) for the preparation of cytospins.

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 cytospins
The two methods used for preparing cytospins 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 cytospins from bone marrow aspirates (data not shown). However, it was determined that at least 3.5 × 10^6 nucleated cells harvested by lysis had to be examined to give an equivalent result to the assessment of 2 × 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 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 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 g for 30 minutes in a temperature controlled centrifuge at 20°C (MSE Mistral 3000i). The cells from theuffy coat at the interface were retrieved, resuspended in PBS, and washed twice by centrifugation at 100 g for five minutes, then resuspended in 5 ml fresh PBS.

Cytospins
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 × 10^5/ïɛtre. Cytospins were made, each from 150 ml (2 × 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 cytospins
Immunohistochemical staining of the cytospins was performed with the anticytokeratin antibodies AE1/AE3 (Dako, Ely, UK), at a concentration of 0.8 µg/ml, and CAM 5.2 (Becton Dickinson, Oxford, UK) at a concentration of 0.5 µg/ml. Briefly, after endogenous peroxidase activity was blocked by immersion for 10 minutes in 3% alcoholic hydrogen peroxide, the cytospins 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 cytospin 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).
Lymph nodes
Representative lymph node stations with no metastases identified on histological review were immunostained with AEI/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 μm onto 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 μg/ml, with overnight incubation. Immunostaining with anti-CD34 mouse monoclonal antibody (Serotec, Kidlington, Oxford, UK) at 10 μg/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.

ASSSESSMENT 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 cytospins 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 mm² 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 ×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 χ² 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 cytospins and nine on smears. Immunopositive cells were identified on the cytospins 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
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 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 immunoreexpression 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 cases where the tumour nm23-H1 immunoreexpression 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
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.

### 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.

### 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 | 3.0 |
| Grade 1 | 7 | 86.1 | 31.6 | 0.090† |
| Grade 2 | 21 | 62.6 | 29.0 | 2.0 |
| Grade 3 | 14 | 53.2 | 34.1 | 3.0 |
| No vascular invasion | 30 | 63.6 | 30.6 | 0.976* |
| Vascular invasion | 12 | 63.9 | 38.3 | 3.0 |
| No lymphatic invasion | 32 | 61.0 | 31.4 | 0.377* |
| Lymphatic invasion | 10 | 71.6 | 35.9 | 3.0 |

MVC was determined in the 42 primary tumours that were assessed for nm23-H1 immunoreactivity. The mean values/standard field (size 0.41 mm²) are shown.

* Student’s t-test; † One way ANOVA [grade 1 v 3; p=0.045; Student-Newman-Keuls test].

**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 node mode of spread.1 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-H2,2 whereas we used an antibody that only detected nm23H-1, because this has been more closely associated with antimetastatic behaviour.3 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 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,4 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.

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References

Severity of meningococcal disease

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 patient's 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 (>8 on Glasgow Meningococcal Septicaemia Prognostic Score) (median load 8.4 × 10^6 severe versus 1.1 × 10^6 mild disease), particularly in septicaemia patients (1.6 × 10^11 versus 9.2 × 10^10, 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 equate 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.

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