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Immunohistochemical Analysis of nm23/NDP Kinase Expression in Human Lung Adenocarcinoma: Association with Tumor Progression in Clara Cell Type

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Levels of nm23-H1/nucleoside diphosphate kinase (NDP kinase) expression have been reported to correlate inversely with metastatic potential in some tumors but not in others. Whether or not nm23 gene product is associated with metastatic potential in lung cancer is not clear as yet. We therefore immunohistochemically examined the expression of nm23 gene products in primary lung adenocarcinomas according to cytologic subtypes in order to clarify the association of its expression with the clinical features of the disease. Seventy-two (64.9%) of the 111 lung adenocarcinomas were positive for nm23 protein. In lung adenocarcinoma of Clara cell type, high levels of nm23 expression were associated with advanced pathologic stage, positive lymph node status, and poorer prognosis (P<0.05). However, no correlation with clinical outcome was observed in other cell types. Our data suggest that higher levels of nm23 expression are associated with tumor progression in lung adenocarcinoma of Clara cell type.

Key words: nm23/NDP kinase — Immunohistochemistry — Lung adenocarcinoma

nm23-H1 gene was identified as a metastasis suppressor gene by differential hybridization of nonmetastatic and metastatic clones of mouse K-1735 melanoma cell line.[1] Expression of the nm23 gene has been associated with low metastatic potential in human tumors[2, 3] and in established cell lines.[1, 4] In breast carcinomas, a high expression of nm23-H1 mRNA was associated with a good prognosis.[5] However, in studies examining colon carcinoma or neuroblastoma, increased nm23-H1 gene expression was found to be associated with advanced stages of the disease.[6, 7] As to lung adenocarcinoma, Higashiyama *et al.*[8] found no relationship between the extent of nm23 gene product expression and the grade of malignancy. Lung adenocarcinoma is a heterogeneous group of diseases with respect to histologic type, cytologic type, and behavior that can be divided into five subtypes identified by their cytologic and ultrastructural features.[9] This absence of correlation of nm23 expression with metastasis could be due to differences in the heterogeneous phenotypes of lung adenocarcinomas.

We therefore immunohistochemically examined the expression of nm23 gene products in primary lung adenocarcinomas according to cytologic subtypes in order to clarify the association of nm23 expression with tumor metastatic potential.

MATERIALS AND METHODS

Patients Tumor specimens were obtained from primary lung adenocarcinomas from 111 patients treated surgically between 1978 and 1992 in our hospital. Fifty-nine patients were male and 52 were female. The age range was 36 to 85 years (mean, 63.0 years). TNM staging was defined according to the General Rules for Clinical and Pathological Recording of Lung Cancer published by the Japan Lung Cancer Society.[10]

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin, and a histologic and cytologic diagnosis was made. Well and moderately differentiated adenocarcinomas were classified cytologically into 5 subtypes according to Shimosato's criteria[9]: bronchial surface epithelial cell type, bronchial gland cell type, goblet cell type, Clara cell type, and type II alveolar epithelial cell type (Table I). All materials were reviewed retrospectively by one pathologist and histologic and cytologic diagnoses were made.

RT-PCR The whole nm23-H1 gene coding regions were amplified by using the following primers: sense-strand primers, H1U, 5′-TGGAAGGATCCATGGCCAACT-GT-3′ (contained a *Bam* HI site): antisense-strand primers, H1D, 5′-GGAGAATTCACAGCTCCAAGA-GC-3′ (contained an *Eco* RI site). All restriction sites included in primers were generated artificially by base substitutions to facilitate later cloning into plasmids. RT-PCR[2] for nm23-H1 yielded a 609-bp product.

Construction of plasmid pGEX-H1 and purification of nm23-H1 protein pGEX-H1 was constructed to express the nm23-H1 protein fused with a 26 kDa GST in *E. coli.*

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[2] The abbreviations used are: RT-PCR, reverse transcriptase-polymerase chain reaction; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

The RT-PCR products from human lung fibroblast RNA were digested with *Bam* HI and *Eco* RI, and cloned into pGEX-2T (Pharmacia). DH 5α *E. coli* was transformed with pGEX-H1, and nm23-H1/GST fusion protein was purified according to the manufacturer's manual using a glutathione-Sepharose 4B column (Pharmacia). Protein purity was confirmed by SDS-PAGE (12%) (Fig. 1a).

Polyclonal antibody against nm23-H1 protein Polyclonal antibody against GST/nm23-H1 fusion protein was raised in a rabbit. nm23-H1 specific antiserum was purified by adsorption with GST protein. Briefly, the extract of DH 5α *E. coli* transformed with pGEX-2T (Pharmacia) was loaded onto a glutathione-Sepharose 4B column. After washing of the column with PBS, antiserum diluted with PBS at 1:100 was passed through the column. The eluted antiserum was assayed for reactivity with nm23-H1 and GST proteins using Western blot analysis (Fig. 1b).

Immunohistochemistry Immunohistochemical staining was also performed on paraffin sections using an avidin-biotinyl peroxidase complex method. Briefly, deparaffinized, rehydrated sections were treated with 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. The slides were preincubated with 10% BSA for 1 h. Excess BSA solution was drained and the sections were incubated with anti-nm23-H1 antibody at a dilution of 1:200 for 1 h. Then, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector) diluted 1: 250 for 30 min, followed by incubation with horseradish peroxidase-conjugated streptoavidin (Vector) at 1:250 in PBS for 30 min. The peroxidase reaction was performed using 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 *M* Tris-HCl buffer, pH 7.4. Finally, nuclear counterstaining was performed with Mayer's hematoxylin. Macrophages in the section served as positive controls. For negative controls for the immunostaining, the primary antibody was replaced with anti-GST polyclonal antibody established in our laboratory.

Immunoreactivity was graded as follows; ++: greater than 30% of cancer cells were stained more intensely than macrophages, +: less than 30% of cancer cells were stained, or the staining intensity of cancer cells was

Table I. Materials

	No. of cases (%)[a]
Well differentiated adenocarcinoma[b]	90
Bronchial surface epithelial type	9 (10.0)
Bronchial gland cell type	19 (21.1)
Goblet cell type	5 (5.6)
Clara cell type	56 (62.2)
Type II alveolar epithelial cell type	1 (1.1)
Poorly differentiated adenocarcinoma	21
Total	111

a) The number and percentage of cases of well-differentiated adenocarcinoma.
b) Including moderately differentiated.

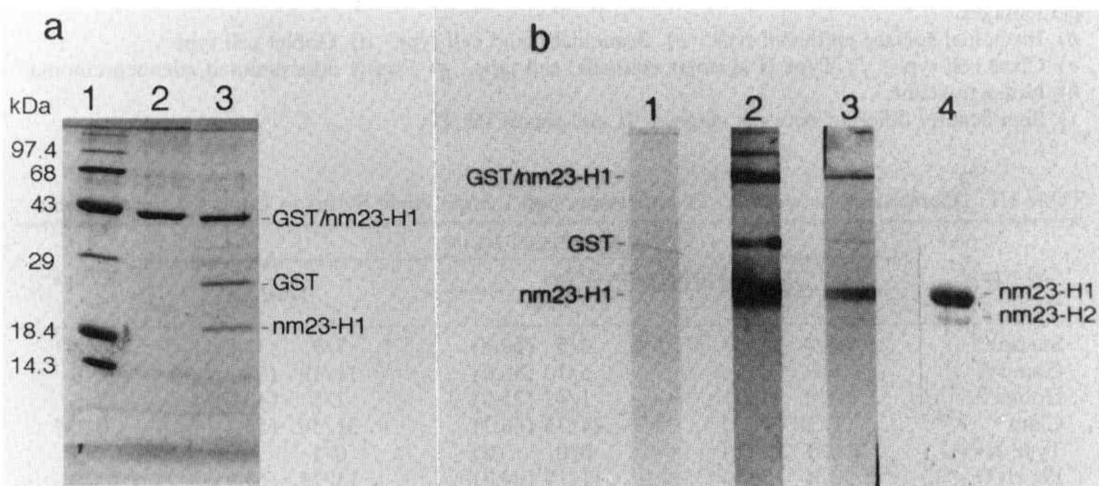

Fig. 1. Analysis of nm23-H1 protein and anti-nm23-H1 antibody. (a) nm23-H1 protein was produced in *E. coli* as a fusion protein with a 26 kDa GST. GST/nm23-H1 fusion protein (lane 2) and thrombin-digested fusion protein were fractionated by SDS-PAGE and stained with Coomassie blue (lane 3). Molecular weight markers are shown in lane 1. (b) Thrombin-digested GST/nm23-H1 fusion protein separated by 15% SDS-PAGE was electrophoretically transferred onto nitrocellulose membrane. The membrane was probed with preimmune rabbit serum (lane 1), antiserum to GST/nm23-H1 fusion protein (lane 2), and antiserum adsorbed with GST protein (lane 3). Western blot analysis of lung fibroblast lysate using antiserum adsorbed with GST protein (lane 4).

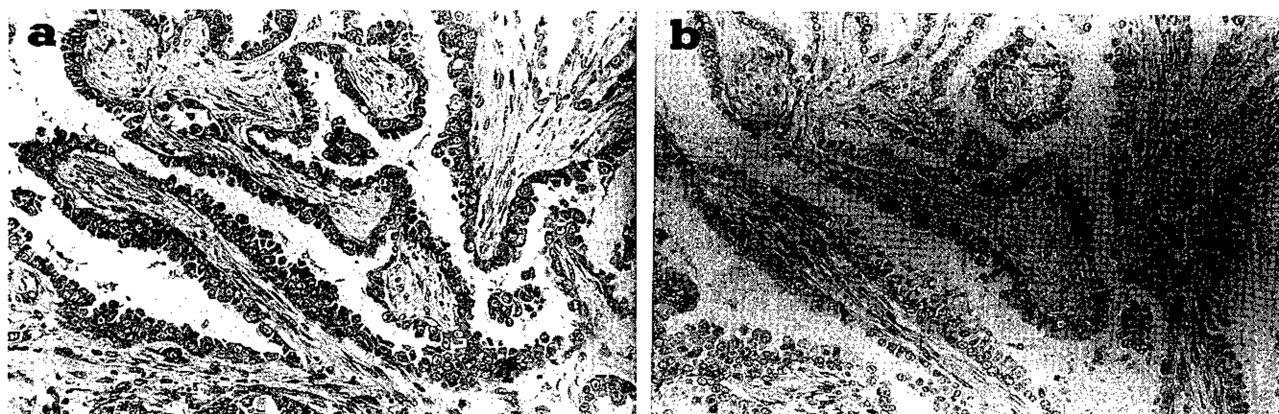

Fig. 2. Immunohistochemical staining for nm23 protein in lung adenocarcinoma (a) (graded as ++). Positive cells showed diffuse cytoplasmic staining. Anti-GST polyclonal antibody was used for negative control staining. (b) No immunoreaction with anti-GST antibody was observed in cancer cells or normal lung tissues. (Serial sections, counterstained with Mayer's hematoxylin, original magnification; ×200)

Table II. Correlation between nm23 Expression and Pathologic Stage in Lung Adenocarcinoma

Cell type	No. of cases (%)[a] Stage I, II	III, IV	Total	P value
Surface[b]	1/2 (50.0)	4/7 (57.1)	5/9 (55.6)	NS[h]
Gland[c]	2/4 (50.0)	8/15 (51.9)	10/19 (52.6)	NS
Goblet[d]	2/2 (100)	2/3 (66.6)	4/5 (80.0)	NS
Clara[e]	12/25 (48.0)	24/31 (77.4)	36/56 (64.3)	<0.05[i]
Type II[f]	0/1 (0)	0/0 (0)	0/1 (0)	NS
Poorly[g]	6/6 (100)	11/15 (73.3)	17/21 (81.0)	NS
Total	23/40 (57.5)	49/71 (69.0)	72/111 (64.9)	NS

a) The numerator represents the number of nm23-positive cases of a particular cytologic type; the denominator represents the number of cases of a particular pathologic stage. Figures in parentheses are percentages.
b) Bronchial surface epithelial type. *c*) Bronchial gland cell type. *d*) Goblet cell type.
e) Clara cell type. *f*) Type II alveolar epithelial cell type. *g*) Poorly differentiated adenocarcinoma.
h) Not significant.
i) Significantly different between stages I, II and stages III, IV.

Table III. Correlation between nm23 Expression and Lymph Node Status in Lung Adenocarcinoma

Cell type	No. of cases (%)[a] nm23 immunoreactivity −	+	Total	P value
Surface[b]	4/4 (100)	3/5 (60.0)	7/9 (77.8)	NS[h]
Gland[c]	6/9 (66.7)	5/10 (50.0)	11/19 (57.9)	NS
Goblet[d]	1/1 (100)	1/4 (25.0)	2/5 (40.0)	NS
Clara[e]	7/20 (35.0)	24/36 (66.7)	31/56 (55.4)	<0.05[i]
Type II[f]	0/1 (0)	0/0 (0)	0/1 (0)	NS
Poorly[g]	2/4 (50.0)	11/17 (64.7)	13/21 (61.9)	NS
Total	20/39 (51.3)	44/72 (61.1)	64/111 (57.7)	NS

a) The numerator represents the number of cases with lymph node metastasis; the denominator represents the number of nm23-positive or negative cases. Figures in parentheses are percentages.
b) Bronchial surface epithelial type. *c*) Bronchial gland cell type. *d*) Goblet cell type.
e) Clara cell type. *f*) Type II alveolar epithelial cell type. *g*) Poorly differentiated adenocarcinoma.
h) Not significant.
i) Significantly different between nm23-positive and negative cases.

similar to that of macrophages, ±: cancer cells were stained less intensely than macrophages, −: cancer cells were not stained. Thus, ++ and + reactions were judged to be positive.

Table IV. Correlation between nm23 Expression and Lymph Node Status According to T Factor in Lung Adenocarcinoma

Cell type	T factor	No. of cases (%)[a] nm23 immunoreactivity −	+	P value
Clara[b]	T 1	2/12 (16.7)	4/11 (36.4)	NS[d] (0.28)[f]
	T 2	1/4 (25.0)	11/14 (78.6)	<0.05[e]
	T 3	4/4 (100)	8/8 (100)	NS
	T 4	− −	1/3 (33.3)	NS
	Total	7/20 (35.0)	24/36 (66.7)	<0.05[e]
Non Clara[c]	T 1	1/2 (50.0)	1/2 (50.0)	NS
	T 2	5/7 (71.4)	8/14 (57.1)	NS
	T 3	2/2 (100)	0/2 (0)	NS
	T 4	3/4 (75.0)	0/2 (0)	NS
	Total	11/15 (73.3)	9/19 (47.4)	NS (0.12)[f]

a) The numerator represents the number of nm23-positive cases of a particular cytologic type; the denominator represents the number of cases of a particular pathologic stage. Figures in parentheses are percentages.
b) Clara cell type.
c) The other cell types of well and moderately differentiated adenocarcinoma (bronchial surface epithelial type, bronchial gland cell type, goblet cell type and Type II alveolar epithelial cell type).
d) Not significant.
e) Significantly different between nm23-positive and negative cases.
f) The *P* value of the chi-square test between nm23-positive and negative cases.

Statistical analysis Statistical significance of differences was evaluated by using the chi-squared test with $P<0.05$ as the criterion of significance. Curves for overall survival were drawn according to the Kaplan-Meier method,[11] and differences between the curves were analyzed by applying the generalized Wilcoxon test.[12] Patients who died of causes other than cancer were excluded from analysis for survival.

RESULTS

Of the 111 primary lung adenocarcinomas, 72 (64.9%) were positive for nm23. Little or no nm23 expression was observed in non-cancerous parts of the lung. Positive cells showed diffuse cytoplasmic staining (Fig. 2a).

The percentage of nm23-positive cases varied according to cytologic type. Only in Clara cell type was nm23 staining significantly correlated with pathologic stage ($P<0.05$) (Table II). The incidence of regional lymph node metastasis in Clara cell type was significantly higher in nm23-positive cases (66.7%) than in negative cases (35.0%) ($P<0.05$) (Table III). In each T group, the incidence of regional lymph node metastasis was also higher in nm23-positive cases than in negative cases in Clara cell type (Table IV). The incidence of distant metastasis was higher in nm23-positive cases (25.0%) than in negative cases (10.0%) ($P=0.16$) (Table V). In all other cell types, no correlation was found between nm23 staining and pathologic stage, lymph node status, or distant metastasis.

There was a significant difference in overall survival curves between the nm23-positive and negative groups ($P<0.05$) (Fig. 3). In the Clara cell type tumors, the nm23-positive group had a poorer prognosis than the

Table V. Correlation between nm23 Expression and Distant Metastatic Status in Lung Adenocarcinoma

Cell type	No. of cases (%)[a] nm23 immunoreactivity −	+	Total	P value
Surface[b]	1/4 (25.0)	1/5 (20.0)	2/9 (22.2)	NS[h]
Gland[c]	3/9 (33.3)	2/10 (20.0)	5/19 (26.3)	NS
Goblet[d]	0/1 (0)	1/4 (25.0)	1/5 (20.0)	NS
Clara[e]	2/20 (10.0)	9/36 (25.0)	11/56 (19.6)	NS (0.16)[i]
Type II[f]	0/1 (0)	0/0 (0)	0/1 (0)	NS
Poorly[g]	1/4 (25.0)	3/17 (17.6)	4/21 (19.0)	NS
Total	7/39 (17.9)	16/72 (22.2)	23/11 (20.7)	NS

a) The numerator represents the number of cases with metastasis in a distant organ; the denominator represents the number of nm23-positive or negative cases. Figures in parentheses are percentages.
b) Bronchial surface epithelial type. *c*) Bronchial gland cell type. *d*) Goblet cell type.
e) Clara cell type. *f*) Type II alveolar epithelial cell type. *g*) Poorly differentiated adenocarcinoma.
h) Not significant.
i) The *P* value of the chi-square test between nm23-positive and negative cases

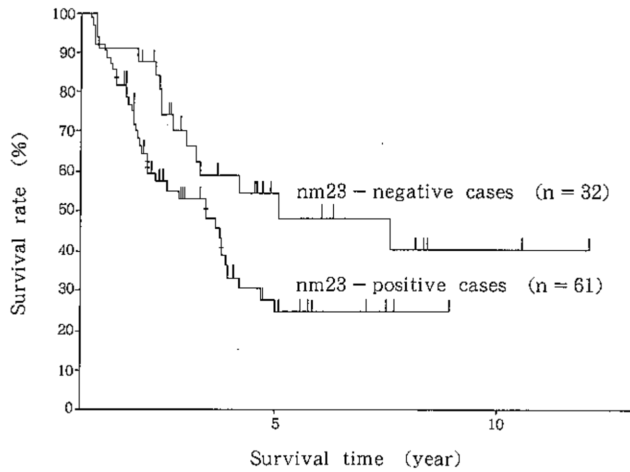

Fig. 3. Kaplan-Meier survival curve of patients with lung adenocarcinoma, with regard to nm23 protein expression. The nm23 protein-positive cases had a poorer survival rate than negative cases ($P<0.05$; generalized Wilcoxon test). Vertical lines indicate surviving patients.

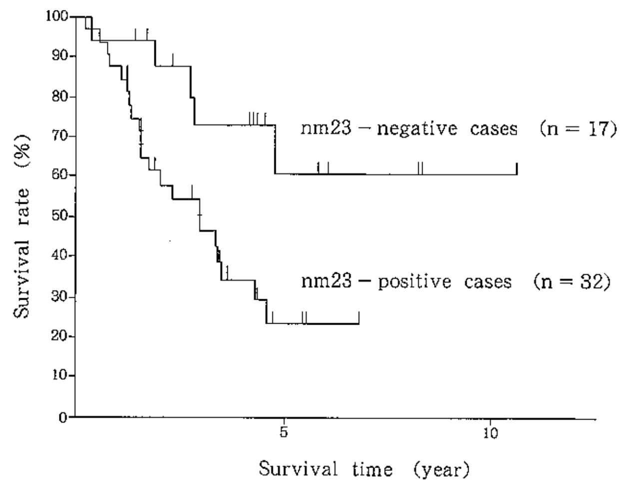

Fig. 4. Kaplan-Meier survival curve of patients with lung adenocarcinoma of Clara cell type, with regard to nm23 protein expression. The nm23 protein-positive cases had a poorer survival rate than negative cases ($P<0.05$; generalized Wilcoxon test). Vertical lines indicate surviving patients.

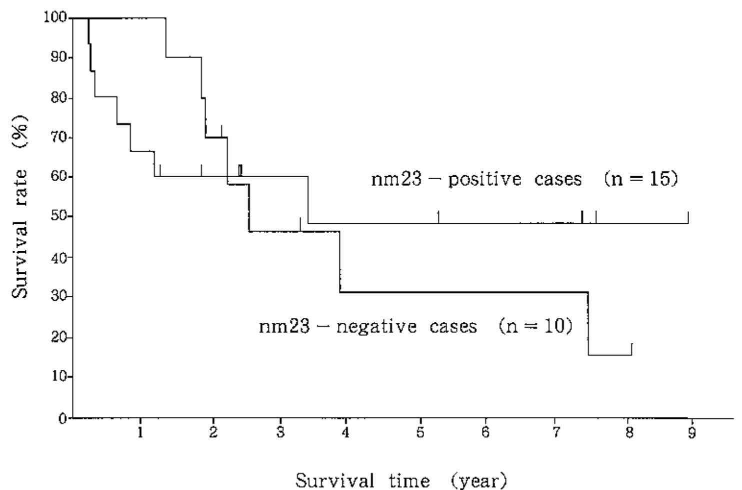

Fig. 5. Kaplan-Meier survival curve of patients with well differentiated lung adenocarcinoma other than Clara cell type with regard to nm23 protein expression. There was no significant difference between nm23-positive cases and negative cases. Vertical lines indicate surviving patients.

negative group ($P<0.05$) (Fig. 4). However, in the other cell types, there was no significant difference in survival rate between the two groups (Fig. 5).

DISCUSSION

nm23-H1 gene was originally identified as a metastasis suppressor gene,[1] and has been found to be inversely associated with metastatic potential in human breast cancer[3, 5, 13] or melanoma.[14] Whether or not nm23 gene product is associated with metastatic potential in lung cancer is not clear as yet. Since lung adenocarcinoma is thought to be a heterogeneous group of diseases with respect to histologic type, cytologic type, and behavior,[9] we immunohistochemically examined the expression of nm23 gene products in primary lung adenocarcinomas according to cytologic subtypes in order to clarify the association of its expression with the clinical features of the disease.

High levels of nm23 protein were associated with advanced pathologic stage, positive lymph node status, and poorer prognosis in lung adenocarcinoma of the Clara cell type, while no correlation was found between nm23 expression levels and clinicopathologic features in other cell types. The results on Clara cell type of lung adenocarcinoma disagree with the previous findings that

nm23 expression is inversely associated with metastatic potential.[1, 3–5, 13, 14] However, results similar to ours have been noted in several other human malignancies. Haut *et al.* have found increased nm23 gene expression in neoplastic colon tissue compared with the levels in normal mucosa from the same individuals.[7] Moreover, Hailat *et al.* have found that high levels of the nm23 protein were associated with advanced stage of the disease in a study on neuroblastoma.[6]

On the other hand, although there was no significant difference in the other cell types of well and moderately differentiated adenocarcinoma, the incidence of lymph node metastasis was higher in nm23-negative cases than in positive cases in each T group (Table IV), and the nm23-negative group had a poorer 5-year survival than the positive group (Fig. 5). These data raise the possibility that nm23 expression levels are inversely associated with cancer progress in these cell types, as in breast cancer.

These discrepancies regarding the significance of nm23 expression in lung adenocarcinoma are not understood, but two possibilities can be considered. First, the biological significance of nm23 expression may be quite different in different cell types of lung adenocarcinoma. Secondly, the levels of expression of nm23-H1 and nm23-H2 may be different in different cell types. In breast tumors, decreases in the level of nm23-H1 mRNA have been found to be more closely associated with a high metastatic potential than decreases in the level of nm23-H2 mRNA.[15] It is still unknown which type is predominant with regard to the metastatic potential of lung cancer. The antibody used in the present study reacts with both types (Fig. 1b).

Although the specific function of NDP kinase is still unknown, recent studies suggest that NDP kinase activates small guanosine triphosphate-binding proteins and participates in biochemical pathways similar to those of the *ras* oncogenes.[16, 17] The NDP kinases are thought to provide intracellular pools of nucleoside triphosphate, to regulate polymerization of microtubules in the mitotic spindle and cytoskeleton, and to supply GTP to G proteins in signal transduction.[18] The possibility remains that nm23, as a kinase that generates GTP, could play a variety of roles depending on other factors which vary between cells and tumors.

We have shown that immunohistochemical staining of nm23 correlated significantly with pathologic stage, regional lymph node status and overall survival in primary lung adenocarcinoma of the Clara cell type. These results suggest that nm23 may play an important role in the progression and dissemination of carcinoma of this cell type as well as in colon carcinoma or in neuroblastoma.

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