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Correlation between nm23 Protein and Several Cell Adhesion Molecules in Human Gastric Carcinoma

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The correlation between nm23 protein (nm23) expression and the expression of several cell adhesion molecules was studied immunohistochemically in 110 resected gastric carcinomas. Formalin-fixed and paraffin-embedded samples were serially sectioned and stained with antibodies against nm23, integrin β_1 subfamily members ($\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_4\beta_1$), LFA-1, ICAM-1, sialyl Lewis^x (sLe^x) and CD44H, -V3, and -V6. Primary carcinomas presenting with either lymph node involvement or liver metastasis expressed significantly reduced levels of nm23 compared to tumors without metastasis. The percent of tumors expressing each adhesion molecule was as follows: $\alpha_2\beta_1$, 27.3%; $\alpha_3\beta_1$, 20.0%; $\alpha_4\beta_1$, 14.5%; LFA-1, 14.5%; ICAM-1, 12.7%; sLe^x, 67.3%; CD44H, 55.5%; CD44V3, 20.0%; and CD44V6, 4.5%. Expression of $\alpha_2\beta_1$ integrin and high levels of sLe^x were significantly correlated with lymph node metastasis, and expression of $\alpha_3\beta_1$ integrin and high levels of sLe^x were correlated with liver metastasis. Expression of ICAM-1 was inversely correlated with liver metastasis. Comparing the expression of each cell adhesion molecule with nm23 immunoreactivity, expression of sLe^x was significantly associated with nm23 expression. Of tumors expressing high levels of sLe^x, 75% showed reduced nm23 expression, compared to 52% of tumors with weak or no sLe^x expression ($P<0.05$). A similar tendency was also observed in the metastasized secondary tumors. These results suggest that reduced nm23 expression may promote the metastatic properties of cancer cells in concert with increased sLe^x expression.

Key words: Gastric cancer — Metastasis — nm23 — Cell adhesion molecule — Sialyl Lewis^x

The nm23 gene was originally identified by differential screening of a murine K1735 melanoma cell line cDNA library using mRNA derived from cell lines with differing metastatic potentials.[1] This gene expression was found to be inversely related to metastatic potential, with 10-fold higher mRNA concentrations in cell clones with low metastatic potential than in clones with high metastatic potential.[1] Leone *et al.*[2] have demonstrated that transfection of the nm23 gene into highly metastatic tumor cells suppressed their metastatic potential. A close relationship between reduced nm23 expression and metastasis has also been reported in several human carcinoma cells.[3–7] Recently, we have studied the expression of nm23 in human gastric carcinomas and have found a similar correlation.[8] These findings have led to the speculation that nm23 has a specific biological role in suppressing tumor metastasis. However, the actual mechanism by which nm23 modifies the metastatic properties of cancer cells is unknown.

Remarkable progress has been made in the detection of various factors that participate in the metastatic process of tumor cells. Among these factors, cell adhesion molecules are thought to play extremely important roles. To clarify the mechanism by which reduced nm23 expression promotes metastatic growth, we studied the correlation between nm23 expression and the expression of several cell adhesion molecules in human gastric carcinomas.

MATERIALS AND METHODS

Tissue samples A hundred and ten surgically resected primary gastric carcinomas and their metastatic lymph node tissues were used for immunohistochemical analysis. The specimens were fixed with 10% buffered formalin (pH 5.9) and embedded in paraffin. The central portion of the tumor was then cut into 4 μm sections. The first sections were routinely stained with hematoxylin and eosin for histologic diagnosis and additional sequential sections were left unstained for immunohistochemistry.

Immunohistochemistry Sections were deparaffinized in xylene, washed with phosphate-buffered saline (PBS) three times each for 5 min, and immersed in 1% hydrogen peroxide in methanol for 30 min in order to block endogenous peroxidase activity. Sections then were washed with PBS three times for 5 min and incubated with 30% normal bovine serum albumin (BSA) at room temperature for 60 min to minimize background staining. Slides from each sample were then incubated with the following antibodies: anti-human nm23 polyclonal antibody (provided by E. Tahara, First Department of Pathology, Hiroshima University), which is known to react

with both nm23 H-1 and nm23 H-2 proteins[5]; anti-human integrin monoclonal antibody (mAb) specific against α_2, α_3 and α_4 subunits, respectively (Chemicon, Temecula, CA); anti-human lymphocyte function associated antigen-1 (LFA-1) mAb (Zymed, San Francisco, CA); anti-human intercellular adhesion molecule-1 (ICAM-1) mAb (Zymed); anti-human sialyl Lewis^x (sLe^X) mAb (Seikagaku, Tokyo); anti-human CD44 hemopoietic mAb (R&D Systems Europe, Abingdon, UK); and anti-CD44 variant mAbs specific against v3 and v6, respectively (R&D Systems Europe). Immunoreactivity was visualized using a commercially available streptavidin-biotin system (HISTOFINE SAB-PO, Nichirei, Tokyo).

Evaluation of immunoreactivity Immunoreactivity of the nm23 antibody was graded as − to +++ according to the number of positive cells and the intensity of the reaction. Each grade was defined as follows: −, almost no positive cells; +, positive cells staining less intensely than the normal gastric mucosa; ++, most positive cells staining as intensely as the normal mucosa; +++, most positive cells staining more intensely than the normal mucosa. Samples with nm23 expression grades of − or + were included in the reduced expression group, and those graded as ++ or +++ were placed into the nonreduced expression group. Immunoreactivity of each cell adhesion molecule was classified into the following 4 grades: −, almost no positive cells; +, more than 10% positive cells with weak immunoreactivity; ++, more than 20% positive cells with moderate immunoreactivity; +++, more than 20% positive cells with strong immunoreactivity.

Statistics Statistical analyses were made using the χ^2 test. Results were considered significant when the P value was less than 0.05. The clinicopathologic features of gastric carcinomas were described in accordance with the General Rules for Gastric Cancer Study (Japanese Research Society for Gastric Cancer, Japan).[9]

RESULTS

Correlation between nm23 expression and metastasis The nm23 expression grades in 110 primary tumors were as follows: −, 7 (6.4%); +, 61 (55.4%); ++, 23 (20.9%); and +++, 19 (17.3%). The incidence of nodal involvement or liver metastasis was significantly higher in the reduced nm23 expression group than in the nonreduced expression group (both $P<0.05$) (Table I).

Cell adhesion molecule expression The proportion of tumors expressing each cell adhesion molecule was as follows: $\alpha_2\beta_1$ integrin, 27.3%; $\alpha_3\beta_1$ integrin, 20.0%; $\alpha_4\beta_1$ integrin, 14.5%; LFA-1, 14.5%; ICAM-1, 12.7%; sLe^x, 67.3%; CD44H, 55.5%; CD44v3, 20.0%; and CD44v6, 4.5%. A significant correlation between the expression of cell adhesion molecules and lymph node metastasis was found only with $\alpha_2\beta_1$ integrin; the incidence of lymph node metastasis was clearly higher in tumors expressing $\alpha_2\beta_1$ integrin than in tumors without $\alpha_2\beta_1$ integrin expression ($P<0.05$) (Table II). Similarly, significant correlations between liver metastasis and the expression of both $\alpha_3\beta_1$ integrin and ICAM-1 were detected. In the case of ICAM-1, its expression was inversely correlated with liver metastasis (Table III). Upon classifying the degree of sLe^x expression into two groups on the basis of immunoreaction intensity (one involved cases showing the grade of − or + and the other showing those of ++ or

Table I. Incidence of Lymph Node and Liver Metastases Based on nm23 Immunoreactivity in Primary Tumors

	nm23 immunoreactivity (%)		P value
	Reduced	Nonreduced	
Node metastasis[a)]	48/68 (70.6)	18/42 (42.9)	0.0224
Liver metastasis	25/68 (36.8)	5/42 (11.9)	0.0277

a) Lymph node metastasis.

Table II. Incidence of Lymph Node Metastasis in Primary Tumors with or without Expression of Cell Adhesion Molecule

Expression of cell adhesion molecule	No. of cases	Incidence of node metastasis (%)	P value
$\alpha_2\beta_1$ integrin			
Negative	80	43 (53.8)	0.0492
Positive	30	23 (76.7)	
$\alpha_3\beta_1$ integrin			
Negative	88	51 (58.0)	0.5270
Positive	22	15 (68.2)	
$\alpha_4\beta_1$ integrin			
Negative	94	57 (60.6)	0.9560
Positive	16	9 (56.2)	
LFA-1			
Negative	94	59 (62.8)	0.2463
Positive	16	7 (43.7)	
ICAM-1			
Negative	96	61 (63.5)	0.0904
Positive	14	5 (35.7)	
CD44H			
Negative	49	29 (59.2)	1.0000
Positive	61	37 (60.7)	
CD44v3			
Negative	88	50 (56.8)	0.2631
Positive	22	16 (72.7)	
CD44v6			
Negative	105	63 (60.0)	1.0000
Positive	5	3 (60.0)	
sLe^x			
Negative	36	20 (55.6)	0.6482
Positive	74	46 (62.2)	

Table III. Incidence of Liver Metastasis in Primary Tumors with or without Expression of Cell Adhesion Molecule

Expression of cell adhesion molecule	No. of cases	Incidence of node metastasis (%)	*P* value
$\alpha_2\beta_1$ integrin			
Negative	80	18 (22.5)	0.1107
Positive	30	12 (40.0)	
$\alpha_3\beta_1$ integrin			
Negative	88	19 (21.6)	0.0160
Positive	22	11 (50.0)	
$\alpha_4\beta_1$ integrin			
Negative	94	26 (27.7)	1.0000
Positive	16	4 (25.0)	
LFA-1			
Negative	94	28 (29.8)	0.2578
Positive	16	2 (12.5)	
ICAM-1			
Negative	96	30 (31.2)	0.0330
Positive	14	0 (0.0)	
CD44H			
Negative	49	16 (32.7)	0.3575
Positive	61	14 (23.0)	
CD44v3			
Negative	88	22 (25.0)	0.4221
Positive	22	8 (36.4)	
CD44v6			
Negative	105	27 (25.7)	0.2428
Positive	5	3 (60.0)	
sLe^x			
Negative	36	7 (19.4)	0.2902
Positive	74	23 (31.1)	

Table IV. Incidence of Lymph Node and Liver Metastases Based on sLe^x Immunoreactivity in Primary Tumors

	sLe^x immunoreactivity (%)		*P* value
	(−)-(+)	(++)-(+++)	
Node metastasis[a)]	32/63 (50.8)	34/47 (72.3)	0.0371
Liver metastasis	12/63 (19.0)	18/47 (38.3)	0.0427

a) Lymph node metastasis.

+++), the incidence of nodal involvement or liver metastasis was significantly higher in tumors with ++ or +++ grades than in tumors with − or + grades (both $P<0.05$) (Table IV). There was no significant correlation between the expression of these cell adhesion molecules and histologic grades (Table V).

Correlation between nm23 and cell adhesion molecule expression A significant correlation was found between nm23 expression and the expression of sLe^x and ICAM-1 (Table VI). Of tumors expressing high levels of sLe^x, 75% showed reduced nm23 expression. In contrast, the proportion of tumors with reduced nm23 expression was lower in tumors expressing ICAM-1 than in those not expressing ICAM-1. No correlation was found between other cell adhesion molecules and nm23 expressions.

Table V. Expression of Cell Adhesion Molecules Based on Histologic Grade

	Histologic grade (%)		*P* value
	Well[a)] (n=59)	Poor[b)] (n=51)	
sLe^x[c)]	30 (50.8)	27 (33.3)	0.0972
ICAM-1	5 (8.5)	9 (17.6)	0.2491
$\alpha_2\beta_1$ integrin	18 (30.5)	12 (23.5)	0.5452
$\alpha_3\beta_1$ integrin	8 (13.6)	14 (27.5)	0.1147

a) Well and moderately differentiated tubular adenocarcinoma, including papillary adenocarcinoma.
b) Poorly differentiated adenocarcinoma, including signet-ring cell carcinoma.
c) Incidence of increased sLe^x expression is shown.

Table VI. Correlation between ICAM-1, sLe^x and nm23 Immunoreactivities

	nm23 immunoreactivity (%)		*P* value
	Reduced	Nonreduced	
sLe^x			
(−)-(+)	33 (52.4)	30 (47.6)	0.0307
(++)-(+++)	35 (74.5)	12 (25.5)	
ICAM-1			
(−)	64 (66.7)	32 (33.3)	0.0144
(+)-(+++)	4 (28.6)	10 (71.4)	

Expression of nm23 and sLe^x in metastatic foci The inverse correlation between nm23 and sLe^x expressions was also found in metastatic tumors (Fig. 1). Among 66 cases with lymph node metastasis, reduced nm23 expression was found in 77% of the metastatic tumors, which was as frequent as that of the primary tumors (73%). Similarly, 52% of the primary tumors and 59% of their lymph node metastases expressed high levels of sLe^x.

DISCUSSION

Leone *et al.*[2] have demonstrated that transfection of nm23 cDNA into highly metastatic murine K-1735 melanoma cell lines significantly reduces their metastatic potential without affecting tumor cell growth, suggesting that nm23 suppresses the malignant process. In agreement with their report, we have found that the incidence of nodal involvement or liver metastasis in human gastric carcinomas was clearly higher in tumors with reduced nm23 expression than in tumors with nonreduced expression. Although the molecular mechanism by which nm23 modifies the metastatic properties of cancer cells remains unknown, there are a few clues about the potential func-

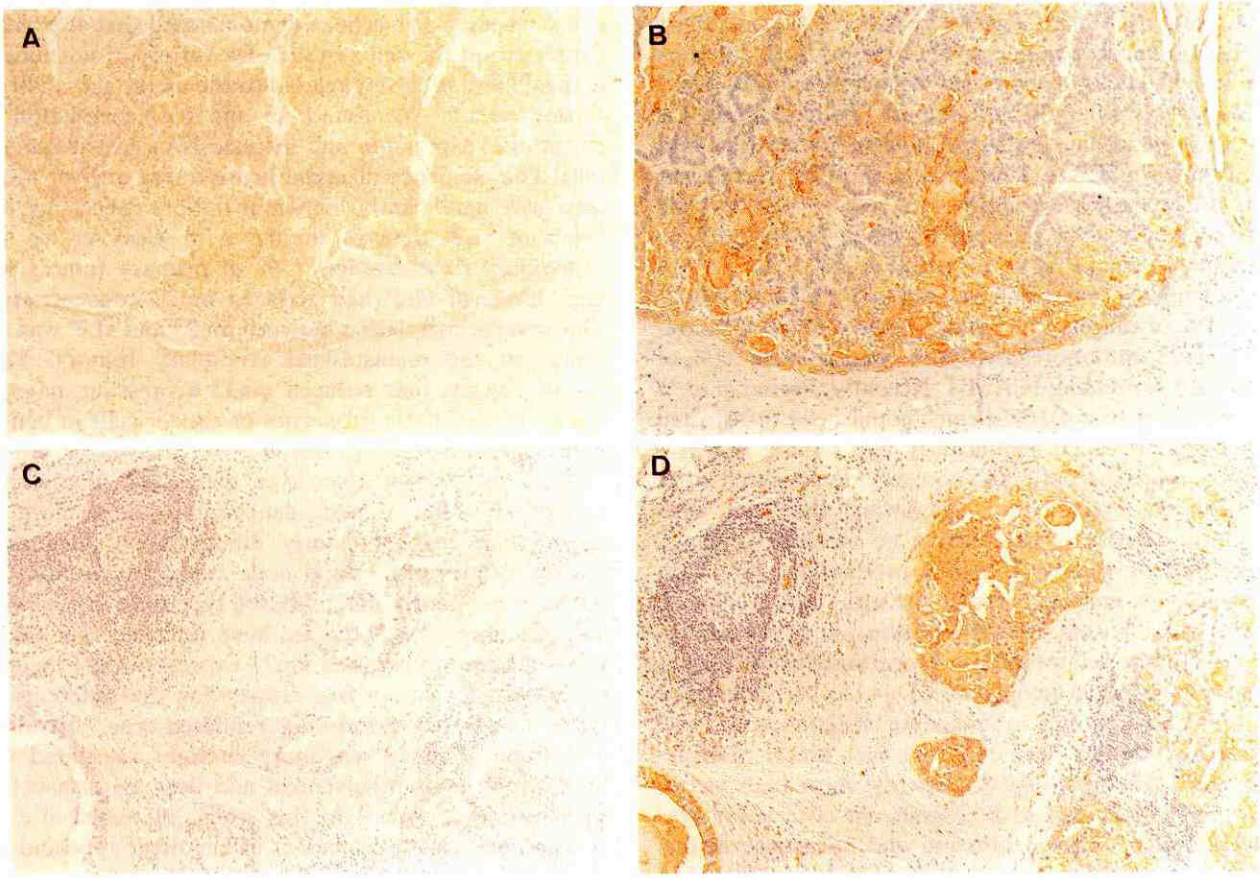

Fig. 1. Immunohistochemical staining of a well differentiated tubular adenocarcinoma of the stomach. The primary tumor shows very weak immunoreactivity for nm23 protein (A) and strong immunoreactivity for sLe^x (B). The metastatic lymph node tumor shows no nm23 expression (C) and high levels of sLe^x (D). ×100.

tion of the nm23 gene product. The amino acid sequence of nm23 shares 78% homology with that of the *Drosophila* abnormal wing disc (*awd*) gene, mutations of which cause abnormal tissue morphology, aberrant differentiation, and cell necrosis during larval development.[10, 11] This raises the possibility that the nm23/*awd* gene participates in normal cell development and that mutation or decreased expression of this gene may induce an unstable developmental state or may facilitate tumor cell metastasis.[12] The nm23 gene product has also been found to share homology with nucleoside diphosphate (NDP) kinase,[13, 14] which is a ubiquitous enzyme catalyzing the transfer of the terminal phosphate of 5′-triphosphate nucleotides to 5′-diphosphate nucleotides by a ping-pong mechanism involving a high-energy phosphorylated enzyme intermediate.[15] NDP kinase has been found to function as a microtubule-associated protein, regulating microtubule assembly and disassembly.[16] Microtubules constitute one of the major components of the cytoskeletal system, influencing the ability of cells to move and adhere. These findings prompted us to study the possible relationship between nm23/NDP kinase and metastasis-related cell adhesion molecules. A correlation between the two might help partially to explain the mechanism by which nm23 modifies the metastatic properties of cancer cells.

Integrin β_1 subfamily members have been identified as extracellular matrix receptors[17] and are known to be associated closely with the cytoskeleton.[18] They are also known to influence the ability of tumor cells to migrate and metastasize.[19, 20] We found that gastric carcinomas expressing $\alpha_2\beta_1$ integrin had a significantly higher incidence of lymph node metastasis than tumors not expressing $\alpha_2\beta_1$ integrin, and that the expression of $\alpha_3\beta_1$ integrin was strongly associated with liver metastasis. Nevertheless, no correlation was found between the expression of these molecules and nm23 expression.

LFA-1 ($\alpha_L\beta_2$ integrin), a receptor for ICAM-1, is usually expressed on activated leukocytes and is involved in T cell proliferation and cytotoxic T lymphocyte

(CTL) activity.[21] Although we considered the possibility that LFA-1 expression may promote tumor cell adhesion to endothelial cells of the target organs through ICAM-1 binding, our results did not support this hypothesis. On the other hand, a significant inverse correlation was found between ICAM-1 expression and liver metastasis. None of the gastric carcinomas expressing ICAM-1 had liver metastasis. This phenomenon may be partially explained by the fact that tumor cells with ICAM-1 expression are highly susceptible to killing by CTL.[22] Moreover, 71% of tumors expressing ICAM-1 showed nonreduced nm23 immunoreactivity, in contrast with 33% of tumors not expressing ICAM-1. Recently, Parhar *et al.*[23] have reported that B16F10 melanoma cells transfected with nm23 produced significantly less soluble ICAM-1 and were more susceptible to lymphokine-activated killer cell-mediated cytotoxicity, suggesting that the antimetastatic activity of nm23 may be mediated through suppression of soluble ICAM-1 production. Although their findings are not consistent with our results, it remains possible that ICAM-1 is involved in the modification of tumor cell metastatic properties by nm23 gene.

The cell surface proteoglycan CD44 has been identified as a molecule involved in leukocyte adhesion and T-cell activation.[24] It has been reported that tumor cells expressing CD44, including its variants, show high metastatic ability.[25] However, no significant correlation was found between CD44 expression and metastasis in this study. Nor was there a correlation between CD44 and nm23 expressions.

The carbohydrate antigen, sLe^x, is usually expressed on the surface of human leukocytes and is involved in leukocyte-endothelial interaction through its binding to E-selectin, which is expressed on activated endothelial cell surfaces.[26] It has been demonstrated that sLe^x is also expressed on the cell surface of a variety of adenocarcinomas[27] and is closely related to the metastatic ability of cancer cells.[28] We found an apparent correlation between sLe^x expression and metastasis in gastric carcinomas. The incidence of nodal involvement or liver metastasis was significantly higher in tumors expressing high levels of sLe^x than in tumors with weak or no sLe^x expression. Furthermore, 75% of primary tumors with high levels of sLe^x had reduced nm23 concentrations. This inverse correlation between nm23 and sLe^x was also found in the metastasized secondary tumors. These results suggest that reduced nm23 expression may promote the metastatic properties of cancer cells in concert with increased sLe^x expression.

It is well-known that liver metastasis of well and moderately differentiated adenocarcinomas is more frequent than that of poorly differentiated adenocarcinomas. Conversely, lymph node metastasis is more frequent in the poorly differentiated type than in the differentiated types. Recently, we have reported that the inverse correlation between nm23 expression and metastasis of gastric cancer was clearer for the differentiated types than for the poorly differentiated type.[8] However, expression of nm23 was more strongly associated with both lymph node involvement and liver metastasis than with histologic type.[8] In this study, increased sLe^x expression was also independent of histologic type and was significantly correlated with lymph node and liver metastases. These results indicate that both nm23 and sLe^x may participate in tumor metastasis independently of histologic features.

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