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Reduced Expression of nm23 Is Associated with Metastasis of Human Gastric Carcinomas

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Reduced expression of nm23 gene is implicated in high metastatic potential in a variety of malignancies. To elucidate the role of nm23 in human gastric carcinomas, we examined loss of heterozygosity (LOH) of nm23 gene by Southern blotting, nm23 mRNA expression by Northern blotting and nm23 protein expression by Western blotting as well as immunohistochemistry in both primary and metastatic tumors. LOH of nm23 gene was found in 2 (8%) out of the 23 informative gastric carcinomas. Twenty-two (84%) out of the 26 cases expressed nm23 mRNA at higher levels in primary tumor tissue than in corresponding non-neoplastic mucosa. No obvious correlation was observed between clinico-pathological features and LOH of nm23 gene or nm23 mRNA expression. On the other hand, 52% of the gastric carcinomas showed reduction of nm23 immunoreactivity in the metastatic tumor of regional lymph nodes, as compared to the primary tumor. Interestingly, 71% of the gastric carcinomas showed weaker nm23 immunoreactivity in the liver metastasis than in the primary tumor. These results suggest that nm23 overexpression is linked with development of gastric carcinomas and the decrease in expression of nm23 participates in metastasis.

Key words: nm23 — Metastasis — Gastric carcinoma

A differential hybridization strategy has been applied to murine K-1735 melanoma cells with low and high metastatic potential, and a novel gene, nm 23, was found.[1] The level of nm23 mRNA is higher in the cells with low metastatic potential.[1] The transfection of nm23 gene into highly metastatic cells resulted in reduced incidence of primary tumor formation and significant reduction in metastatic potentials.[2] Subsequently, two distinct human nm23 genes were isolated as nm23-H1, and -H2, both of which encode proteins of approximately 17 kDa that exhibit a 90% amino acid sequence identity.[3] Recently, it has been shown that nm23-H1 and -H2 are identical to human nucleotide diphosphate (NDP[2]) kinase-A and -B, respectively.[4]

In breast carcinomas, nm23 expression at either mRNA or protein level is associated with good prognosis and a lack of lymph-node metastasis.[1, 5, 6] On the other hand, although expression of nm23 gene increases during the early stage of colon carcinogenesis, allelic deletion of nm23-H1 is correlated with distant metastasis of colon carcinomas.[7] We have recently found that reduced expression of nm23 protein in colorectal carcinomas is associated with tumor stage and distant metastasis.[8] However, there has been no report on genetic alteration and expression of nm23 in human gastric carcinomas.

In this study, we examined loss of heterozygosity (LOH) of nm23 gene by Southern blotting, nm23 mRNA expression by Northern blotting and nm23 protein expression by Western blotting as well as immunohistochemistry in both primary and metastatic tumors, to elucidate the role of nm23 in the development and progression of human gastric carcinomas.

MATERIALS AND METHODS

Gastric tissues and cell lines A total of 26 snap-frozen surgically resected human gastric carcinoma tissues and corresponding non-cancerous mucosae were used for Northern blot analysis. Twenty-five surgically resected primary gastric carcinomas and their metastatic lymph node tissues and 47 autopsy (performed within 3 h after death) cases of paired primary gastric carcinoma tissues and metastatic lesions (liver and distant lymph nodes) were used for immunohistochemical analysis. Both primary and metastatic tumors from individual surgical or autopsy cases were fixed with the same phosphate-buffered 4% formalin solution to minimize the variation of protein degradation within the primary tissue and metastatic foci. TMK-1 cell line (poorly differentiated adenocarcinoma) was established in our laboratory. KATO-III cell line (signet ring cell carcinoma) was kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo). HSC-39 cell line (signet ring cell carcinoma) was kindly provided by Dr. K. Yanagihara (Hiroshima University, Hiroshima). The other five cell

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[2] The abbreviations used are: nm23, non-metastatic 23; NDP, nucleotide diphosphate kinase; LOH, loss of heterozygosity; kb, kilobase.

lines (MKN-1, adenosquamous carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinomas; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima).

Southern blot analysis After treatment with sodium dodecyl sulfate (SDS) and proteinase K, high-molecular-weight DNAs were prepared by using the phenol-chloroform-isoamyl alcohol method. DNAs were digested with *Bgl*II, and 10 μg of completely digested DNAs was electrophoresed on 0.8% agarose gel.[9] DNAs were transferred to a nitrocellulose filter according to the methods of Southern.[10] The filter was hybridized with ^{32}P-labeled nm23-H1 cDNA.[11] After washing, filters were exposed to Kodak XAR-5 films. Human pNM23-H1 and -H2 was kindly provided by Dr. S. Steeg (NIH).

Northern blot analysis RNA was extracted by the guanidium isocyanate/cesium chloride method. Total RNA (10 μg) was electrophoresed on 1.0% agarose/formaldehyde gel and blotted on zeta-probe nylon filter membrane (Bio-Rad Laboratories). Hybridization, washing, and autoradiography were performed as described previously.[12]

Western blot analysis Frozen tissues were homogenized in HEPES-monothio buffer as described previously.[13] Tissue homogenates or cell suspensions were lysed in lysis buffer containing 50 m*M* Tris-HCl (pH 7.4), 125 m*M* NaCl, 0.1% (v/v) NP40, 5 m*M* EDTA, 50 m*M* NaF, 1 m*M* PMSF and 2 μ*M* each of leupeptin, pepstatin and antipain. Lysates were subjected to Western blot analysis as described previously.[13] Anti-nm23 polyclonal antibody was raised against a synthetic oligopeptide corresponding to nucleotide positions 354 to 404 (Peptide 11) of human nm23 cDNA in our laboratory. This antibody reacts with 17 kDa and 18.5 kDa forms of nm23 protein, and the reaction was abolished by preincubating the antibody with an excess of the antigenic oligopeptide (Fig. 1). The antibody recognizes products from both nm23-H1 and -H2 genes, because the sequences corresponding to that of synthetic antigenic peptide (Peptide 11) are identical in nm23-H1 and nm23-H2.

Immunohistochemistry The immunoglobulin enzyme bridge technique (indirect method) was employed.[13] Dewaxed formalin-embedded tissues were subjected to methanol blocking, followed by primary reaction using anti-nm23 antibody. The immunoreactivity in the tissues was graded as − to ++++ according to the number of cells stained and the intensity of the reaction in individual cells. Grades were defined as follows: −, almost no positive cells; +, a few tumor cells positive; ++, small numbers of tumor cells show positive immunoreactivity; +++, large numbers of positive tumor cells showed moderate immunoreactivity and/or small numbers of positive tumor cells showed strong immunoreactivity; ++++, many tumor cells showed strong immunoreactivity. In deciding the grades in primary lesions, we neglected mucosal areas, which sometimes showed non-specific reactions. According to this grading protocol, three independent pathologists (H.N., H.Y., and W.Y.) examined all the immunostained specimens randomly to make the grading as objective as possible. After deciding the grades randomly, we compared the grades of immunoreactivity in primary lesions with those in metastatic lesions. The specificity of immunoreactivity was confirmed by preabsorption of nm23 antibody with an excess of the antigenic oligopeptide (Peptide 11).

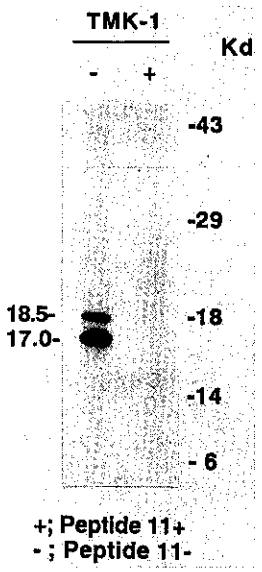

Fig. 1. Competition assay of anti-nm23 antibody in Western blotting. TMK-1 cell lysate (50 μg) was subjected to 15% SDS-PAGE, followed by electrotransfer onto nitrocellulose. Immunoreaction was done with anti-nm23 antibody (Peptide 11−) or the antibody preincubated with 100-fold molar excess of the antigenic peptide (Peptide 11+).

RESULTS

LOH of nm23-H1 gene A *Bgl*II restriction fragment length polymorphism (RFLP) of human chromosomal DNA, which identified nm23-H1 allelic bands at 2.3 and 7.6 kb, was used for the analysis of LOH of nm23-H1 in human gastric carcinomas. A total of 43 paired DNA samples from matched normal tissue and carcinoma were analyzed. As shown in Fig. 2, only 2 (8%; well differentiated tubular adenocarcinoma and poorly differentiated adenocarcinoma, medullary type) out of the 23 informative tumors exhibited a deletion of one nm23-H1 allele.

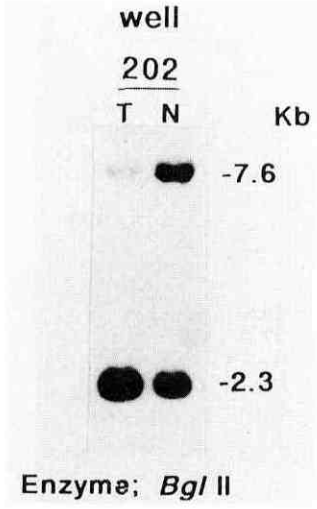

Fig. 2. Allelic loss of nm23 gene in a gastric carcinoma. *Bgl*II-digested DNA (10 μg) was electrophoresed and transferred to Nylon membrane filter. Southern blot analysis using nm23-H1 probe was performed as described in "Materials and Methods." T: DNA from tumor tissue, N: DNA from non-cancerous gastric mucosa.

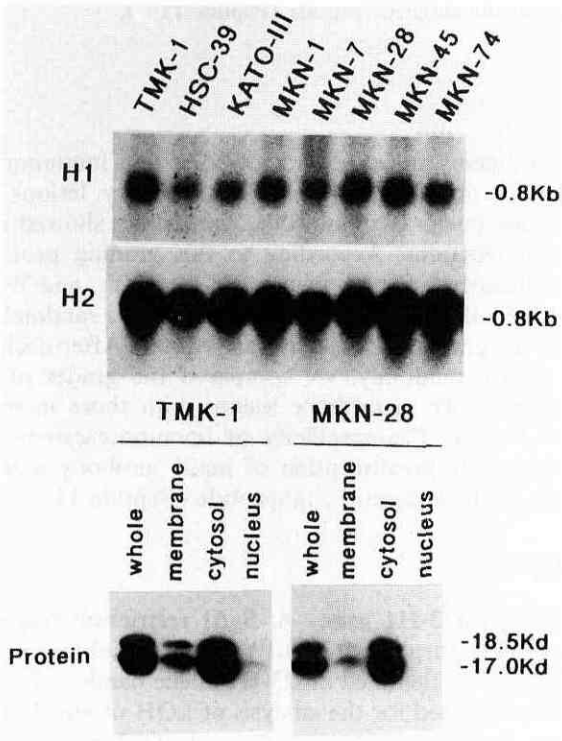

Fig. 3. Expression of nm23 in human gastric carcinoma cell lines. Total RNA (10 μg) was subjected to Northern blot analysis using nm23-H1 and -H2 probes. For nm23 protein localization, subcellular fractionation was performed on TMK-1 and MKN-28, and 50 μg of each fraction was subjected to Western blot analysis.

nm23 mRNA expression Both nm23-H1 and -H2 mRNA were expressed as a 0.8 kb transcript in all the 8 gastric carcinoma cell lines with small variations in the signal intensities (Fig. 3). All the surgically resected gastric carcinomas as well as their paired non-cancerous mucosa also expressed nm23 mRNA. Twenty-two (85%) out of the 26 primary tumors expressed higher levels of nm23 mRNA than their paired non-cancerous mucosae (Fig. 4). As we performed hybridization with the full length nm23-H1 and nm23-H2, differential expression of nm23-H1 and nm23-H2 could not be detected. No obvious correlation was found between nm23 mRNA expression and histological types of gastric carcinoma.

nm23 protein expression We examined the expression of nm23 protein in gastric carcinomas by Western blotting. As shown in Fig. 3, two bands of 17.0 kDa and 18.5 kDa were detected in TMK-1 and MKN-28 cells by anti-nm23 antibody. Both bands were confirmed to be specific by competition assay with the oligopeptide (Peptide 11) used as an immunogen (see Fig. 1). nm23 protein was localized preferentially in the cytosol fraction. Examination of whole tissue extracts of surgically resected tissues gave results compatible with those of Northern blot analysis except for Case 508 (Fig. 4).

Next, we examined the relationship between nm23 immunoreactivity and metastasis of gastric carcinoma. Table I shows a comparison of nm23 immunoreactivity in primary tumor and regional lymph node metastasis in surgically resected cases. Out of the 25 cases examined,

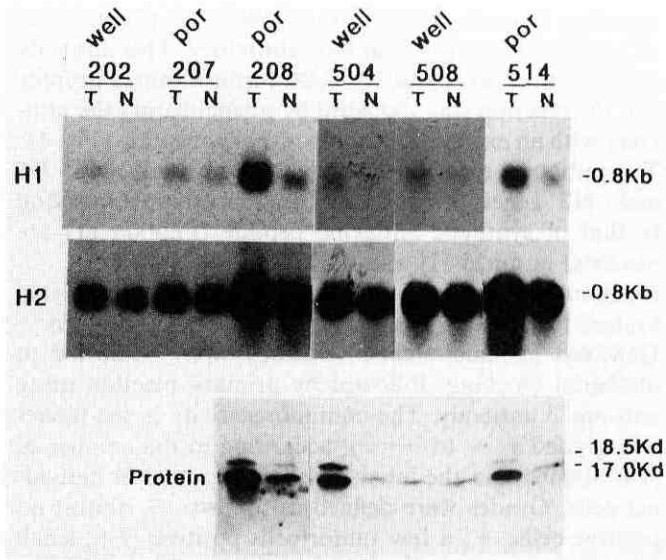

Fig. 4. Expression of nm23 in human gastric carcinoma tissues. Total RNA (10 μg) and whole tissue lysate (50 μg) were subjected to Northern blot and Western blot analysis, respectively.

Table I. Comparison of nm23 Immunoreactivity in Primary Tumors and Regional Lymph Node Metastasis: Surgical Cases

Histology	Number of cases	Positive cases[e]		nm23 immunoreactivity +	++	+++	++++
Well[a]	14	Pr[c]	11 (78.5%)	2	4	1	4
		LN[d]	7 (50.0%)	3	1	0	3
Poorly[b]	11	Pr	10 (90.9%)	2	2	0	6
		LN	7 (63.6%)	2	2	1	2
Total	25	Pr	21 (84.0%)	4	6	1	10
		LN	14 (56.0%)	5	3	1	5

Histology	nm23 immunoreactivity		
	Pr>LN	Pr=LN	Pr<LN
Well	6 (54.5%)	3 (27.3%)	2 (18.2%)
Poorly	5 (50.0%)	4 (40.0%)	1 (10.0%)
Total	11 (52.4%)	7 (33.3%)	3 (14.3%)

a) Well; Well and moderately differentiated tubular adenocarcinoma, including papillary adenocarcinoma.
b) Poorly; Poorly differentiated adenocarcinoma.
c) Pr; Primary tumor.
d) LN; Lymph node.
e) Positive cases showed nm23 immunoreactivity in either primary or metastatic lesions.

primary tumors of 21 (84%) cases showed positive immunoreactivity to nm23 within tumor cells (Fig. 5). When the immunoreactivity of the primary tumors was compared with that of lymph node metastasis, 52% of the cases showed weaker nm23 immunoreactivity in lymph node metastasis.

To elucidate the relationship between nm23 immunoreactivity and distant metastasis of gastric carcinomas, we analyzed autopsy cases (autopy had been performed within 3 h after death). As shown in Tables II and III, the incidence of cases with positive immunoreactivity to nm23 was 52–56%, being lower than in the surgical cases. This might be accounted for by degradation of nm23 protein. In the cases with liver metastasis, 71% of the cases showed weaker nm23 immunoreactivity in metastatic tumor of the liver than in primary tumors, whereas only 6% of the cases showed higher immunoreactivity in the metastatic tumor. As to distant lymph node metastasis, similar findings were observed: 59% of the cases showed reduced nm23 immunoreactivity in metastatic tumors in comparison with primary tumors. However, in contrast to regional lymph node metastasis, nm23 immunoreactivity differed according to histological types. More than 80% of well differentiated adenocarcinoma cases showed a reduction of nm23 immunoreactivity in metastatic tumors of distant lymph nodes (Fig. 6), while

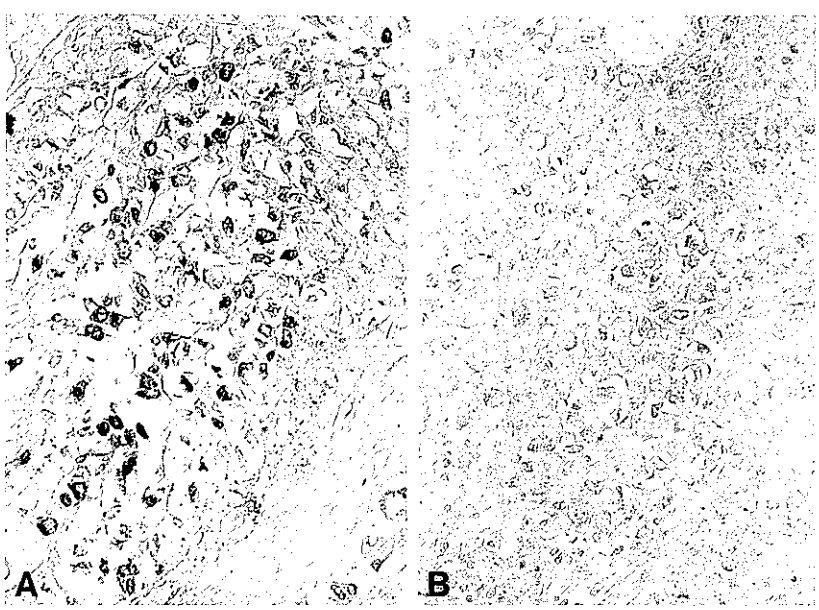

Fig. 5. Photomicrographs of representative surgical case. Case 207. Poorly differentiated adenocarcinoma. (A) Primary tumor, (B) Metastatic tumor. nm23 immunoreactivity in the primary lesion (graded as ++++) was much stronger than that in the metastatic lymph node (−). nm23 immunoreactivity was observed in the primary lesion in both cytoplasm and nucleus of the tumor cells.

Table II. Comparison of nm23 Immunoreactivity in Primary Tumors and Liver Metastasis

Histology	Number of cases	Positive cases[e]		nm23 immunoreactivity +	++	+++	++++
Well[a]	21	Pr[c]	11 (52.3%)	4	4	3	0
		Li[d]	6 (28.6%)	3	3	0	0
Poorly[b]	12	Pr	6 (50.0%)	1	2	2	1
		Li	5 (41.7%)	2	1	1	1
Total	33	Pr	17 (51.5%)	5	6	5	1
		Li	11 (33.3%)	5	4	1	1

Histology	nm23 immunoreactivity		
	Pr>Li	Pr=Li	Pr<Li
Well	8 (72.7%)	3 (27.3%)	0
Poorly	4 (66.6%)	1 (16.7%)	1 (16.7%)
Total	12 (70.6%)	4 (21.5%)	1 (5.9%)

a) Well; Well and moderately differentiated tubular adenocarcinoma, including papillary adenocarcinoma.
b) Poorly; Poorly differentiated adenocarcinoma.
c) Pr; Primary tumor.
d) Li; Liver metastasis.
e) Positive cases showed nm23 immunoreactivity in either primary or metastatic lesions.

Table III. Comparison of nm23 Immunoreactivity in Primary Tumors and Lymph Node Metastasis

Histology	Number of cases	Positive cases[e]		nm23 immunoreactivity +	++	+++	++++
Well[a]	21	Pr[c]	11 (52.4%)	4	4	3	0
		LN[d]	6 (28.6%)	5	1	0	0
Poorly[b]	18	Pr	8 (44.4%)	0	3	3	2
		LN	9 (50.0%)	3	2	3	1
Total	39	Pr	19 (48.9%)	4	7	6	2
		LN	15 (38.5%)	8	3	3	1

Histology	nm23 immunoreactivity		
	Pr>LN	Pr=LN	Pr<LN
Well	10 (83.4%)	1 (8.3%)	1[f] (8.3%)
Poorly	3 (30%)	3 (30%)	4[f] (40%)
Total	13 (59.1%)	4 (18.2%)	5 (22.7%)

a) Well; Well and moderately differentiated tubular adenocarcinoma, including papillary adenocarcinoma.
b) Poorly; Poorly differentiated adenocarcinoma.
c) Pr; Primary tumor.
d) LN; Lymph node.
e) Positive cases showed nm23 immunoreactivity in either primary or metastatic lesions.
f) One well differentiated adenocarcinoma and two poorly differentiated adenocarcinoma cases did not show nm23 immunoreactivity in primary lesions.

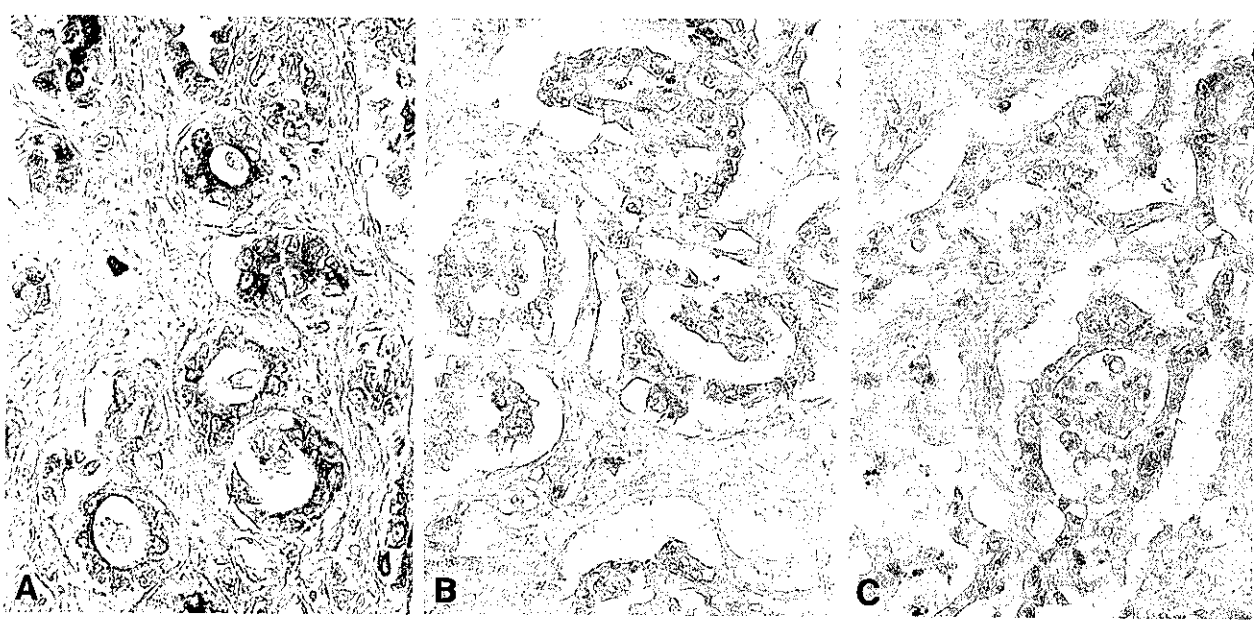

Fig. 6. Photomicrographs of representative autopsy case. Case 1670. Well differentiated adenocarcinoma. (A) Primary tumor, (B) Metastatic liver tumor, (C) Metastatic lymph node tumor. The primary tumor expressed higher levels of nm23 protein (graded as ++++) than did the metastatic liver (−) and lymph node (−) tumors. The primary and metastatic lesions were both obtained at autopsy and fixed simultaneously.

nm23 immunoreactivity in poorly differentiated adenocarcinomas was not different between primary and metastatic tumors.

DISCUSSION

The nm23 gene has been proposed to be a metastasis suppressor gene.[1] Introduction of nm23 expression vector into highly metastatic cells results in a reduction in the incidence of primary tumor formation and of metastatic potential.[2] nm23-H1 and -H2 genes encode human NDP kinase A and B, respectively.[4] LOH of nm23-H1 gene has been reported in carcinomas of the breast, lung, kidney and colorectum.[14] Especially in colorectal carcinomas, LOH of nm23-H1 is correlated with distant metastasis.[6] In the present study, we found only two cases to have LOH of nm23-H1 but they did not show any special clinicopathological features.

In breast carcinomas, high expression of nm23 mRNA and immunoreactivity to nm23 are associated with low metastatic potential and good prognosis.[5,6] The present study on gastric carcinomas also yielded consistent results on mRNA and protein expression. Furthermore, immunohistochemical observation revealed that reduction of nm23 immunoreactivity was associated with metastasis in either regional lymph node, liver, or distant lymph node. In this study, both primary and metastatic tumors from autopsy materials were simultaneously fixed with identical procedures, and the variations of protein degradation should have been minimal. nm23 might participate in metastasis of gastric carcinomas through both lymph vessels and veins. However, as shown in Table III, most of the well differentiated tumors showed a reduction of nm23 immunoreactivity between primary tumors and metastasis, indicating that nm23 is not the only factor regulating metastasis of gastric carcinomas. In fact, activation of oncogenes and overexpression of growth factor/receptor are frequently associated with distant metastasis of gastric carcinomas.[15,16]

nm23 gene encodes NDP kinase. NDP kinase provides intracellular pools of nucleotide triphosphate, regulating polymerization of microtubules in the mitotic spindle and cytoskeleton, and supplies GTP to G-protein in signal transduction.[11] The predicted amino acid sequence of nm23 suggests that nm23 acts as a transcription factor.[17,18] On the other hand, metastasis is a cascade of linked sequential steps involving multiple host-tumor interactions. To form metastatic foci successfully, a tumor cell or group of cells must be able to 1) leave the primary tumor, 2) invade local stroma of host tissue, 3) enter the circulation, 4) attach at a distant vascular bed, 5) extravasate into parenchyma of the target organ, and 6) proliferate to form a second tumor.[19-21] The process with which nm23 is closely associated needs to be identified.

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