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Immunohistochemical analysis of nm23 gene product in human gallbladder carcinomas

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Abstract The expression of nm23, the product of a candidate suppressor gene for tumour metastasis, was examined immunohistochemically in human gallbladder carcinomas and compared with clinicopathological features. Seventy-eight (72%) of 107 carcinomas expressed nm23 protein regardless of histological type, while non-neoplastic mucosa occasionally showed very weak immunoreactivity to nm23. No obvious correlation was observed between nm23 protein expression and depth of tumour invasion or tumour stage. The expression of nm23 protein was detected in 60% and 74% of the cases with and without lymph node metastasis, respectively, indicating no relationship to metastatic ability. Fifty-eight percent of the cases showed reduction of nm23 immunoreactivity in tumour cells invading the stroma at the border of tumour cell nests compared with cells at the centre of the tumour. Only 7% of the cases showed increased nm23 expression in tumour cells at the border. These results suggest that in gallbladder carcinoma decreased expression of nm23 may not have implications for metastasis but may play a part in local invasion.

Key words Nm23/NDP kinase · Gallbladder carcinoma · Immunohistochemistry

Introduction

The nm23 gene was identified from murine K-1735 melanoma cells with low and high metastatic potential as a candidate suppressor gene for tumour metastasis, using a differentiated hybridization strategy [16]. Subsequently, two distinct human nm23 genes were isolated as nm23-H1, and H2, both of which encode proteins of 17–18 kDa that exhibit a 90% amino acid sequence identity [15]. It has been shown that nm23 H1 and H2 are identical to human nucleotide diphosphate kinase-A and -B [3]. The overexpression of nm23 gene in highly metastatic cells by gene transfer reduces metastatic tumour formation. In breast carcinomas, nm23 expression at either mRNA or protein level is associated with good prognosis and lack of lymph node metastasis [5, 7]. We have found that reduced expression of nm23 is associated with gastric and colorectal carcinomas of advanced stage and high metastatic potential [2, 11]. More recently, nm23 H2 has been shown to be identical to c-*myc* transcription factor (PuF) [12]. We have also confirmed that the level of nm23 expression in the gastric and colorectal carcinomas is significantly higher than that in non-neoplastic counterparts [2, 11]. Therefore, nm23 may participate in both development of tumours and suppression of metastasis.

Mutations of K-*ras* oncogene and p53 tumour suppressor genes occur frequently in gallbladder carcinomas [8, 17]. However, little is known about the molecular mechanism of tumour progression and metastasis of these malignancies. There have been no reports on the expression of nm23 and its significance in gallbladder carcinomas.

In this study, in order to elucidate the role of nm23 in metastasis or progression of gallbladder carcinomas, we examined nm23 protein expression using anti-nm23 antibody and compared it with clinicopathological findings.

Materials and methods

A total of 107 surgically resected gallbladder carcinomas and metastatic lesions from regional lymph node, with corresponding non-neoplastic mucosa from the same patients were used. They were formalin-fixed and paraffin-embedded. Both primary tumour and lymph node metastasis were examined in 22 cases. In some

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cases, tumour tissues and non-neoplastic mucosa were frozen in liquid nitrogen immediately after surgery and stored at –80°C. The definition of histological classification and stage grouping were made according to the criteria of the World Health Organization [1] and the International Union against Cancer [19]. Informed consent was obtained from all subjects.

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 [11, 14]. This antibody reacts with 17.0 kDa and 18.5 kDa forms of nm23 protein. The antibody recognizes products from both nm23 H1 and H2 gene, because the sequences corresponding to peptide 11 are identical in human nm23 H1 and H2. The specificity of immunoreaction was confirmed by a pre-incubation with an excess of the antigenic peptides of nm23.

The immunoglobulin enzyme bridge technique (indirect method) was employed [9]. Dewaxed tissue sections were subjected to methanol blocking, following by primary reaction using anti-nm23 antibody. The immunoreactivity 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; +, 10–30% of tumour cells showed weak to moderate immunoreactivity; ++, 30–60% of tumour cells showed weak to moderate immunoreactivity or 10–30% of tumour cells showed strong immunoreactivity; +++, over 60% of tumour cells showed strong immunoreactivity. Three independent pathologists (K.F., W.Y. and F.S.) examined all the immunostained specimens randomly to make the grading as objective as possible. After deciding on the grades, we compared the grades of immunoreactivity in primary lesions with those in metastatic lymph nodes.

For Western blot analysis, frozen tissues were homogenized in HEPES-monothio buffer as described previously [9]. Tissue homogenates were lysed in buffer containing 50 mM TRIS-HCl (pH 7.4), 125 mM NaCl, 0.1% (v/v) NP40, 5 mM EDTA , 50 mM NaF, 1 mM PMSF and 2 μM each of leupeptin, pepstatin and antipain. Lysates were subjected to Western blotting as described elsewhere [9].

The statistical analysis was performed using the Kendall Tau b correlation analysis and signed rank test.

Results

Of 107 gallbladder carcinomas, 78 (72%) showed positive immunoreactivity to nm23 protein, regardless of histological type and grade (Table 1, Fig. 1). The incidence of nm23-positive cases was slightly lower in adenosquamous carcinomas than in adenocarcinomas, but the difference was not significant. Nm23 protein was observed mainly in the cytoplasm of the tumour cells. Non-neoplastic epithelial cells occasionally showed very weak immunoreactivity to nm23.

We next examined the correlation between the expression of nm23 protein and the clinicopathological fea-

Table 1 Immunohistochemical detection of nm23 in human gallbladder carcinoma (The statistical analysis was performed using Kendall Tau b correlation analysis. A probability of $p<0.05$ was considered statistically significant. Significant correlation was not detected ($p=0.3626$)

Histological type [1]	Positive cases	nm23 immunoreaction		
		+	++	+++
pap	17/21 (80%)	9	5	3
well	36/49 (73%)	18	16	2
mod	12/17 (70%)	7	3	2
por	8/11 (77%)	3	5	0
as	5/9 (55%)	3	1	1
Total	78/107 (72%)	40	30	8

Fig. 1a–c Immunostaining of nm23 protein in human gallbladder carcinomas. **a** Poorly differentiated adenocarcinoma. Many tumour cells are positive to nm23 protein within the cytoplasms. ×100. **b** Moderately differentiated adenocarcinoma. ×200. **c** Papillary adenocarcinoma. ×200

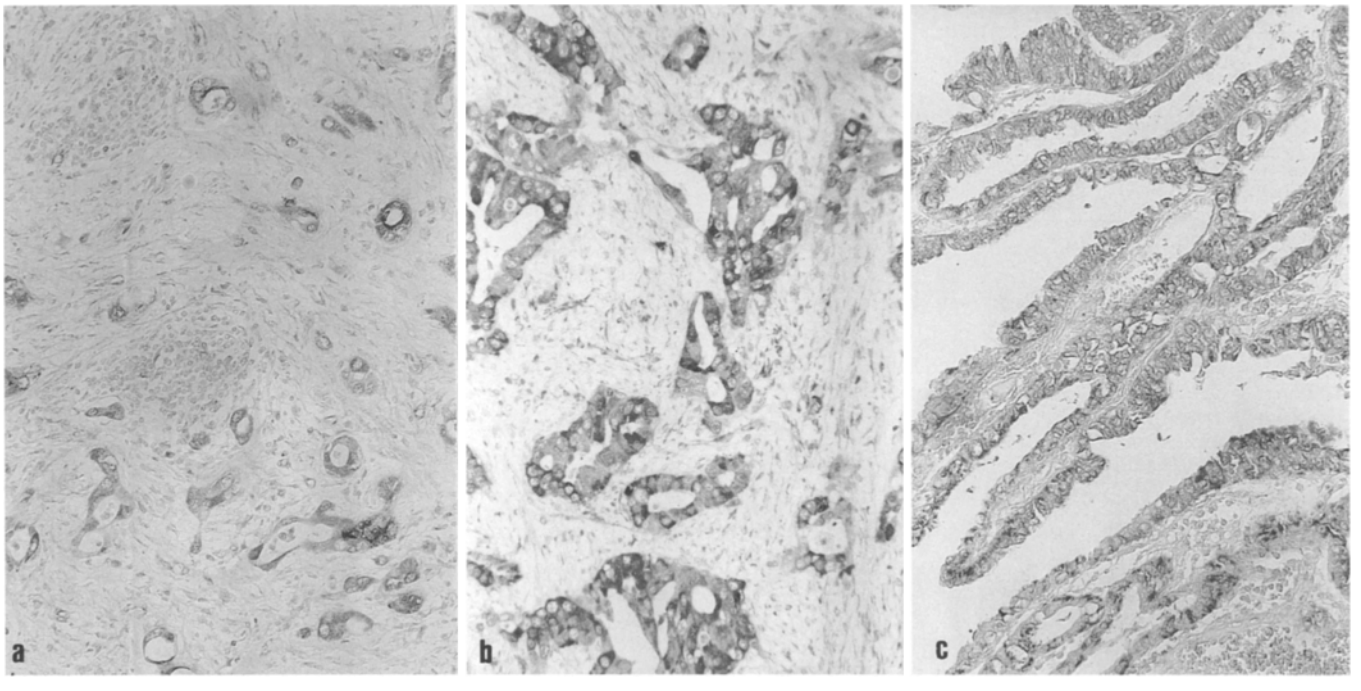

Table 2 Expression of nm23 protein in gallbladder carcinomas with or without lymph-node metastasis

	Positive cases	nm23 immunoreaction		
		+	++	+++
Cases without LN metastasis	12/20 (60%)	5	5	32
Cases with LN metastasis	66/87 (74%)	35	25	6
Total	78/107 (72%)	40	30	8

Table 3 Comparison of nm23 immunoreactivity between primary tumours and lymph-node metastasis

Total cases	Pri>LN	Pri=LN	Pri<LN
22	8 (36%)	5 (23%)	9 (41%)

tures. No obvious correlation was observed between nm23 immunoreactivity and depth of tumour invasion or tumour stage (data not shown). Furthermore, the expression of nm23 protein was not correlated with metastatic ability of the tumour cells. Nm23 protein expression was detected in 60% and 74% of the cases with and without lymph-node metastasis, respectively (Tables 2, 3). When the immunoreactivity of the primary tumours was compared to that of lymph-node metastases, 8 (36%) of the 22 cases available to evaluate showed weaker nm23 immunoreactivity in lymph-node metastasis than the primary tumour, while 9 (41%) showed higher immunoreactivity in the metastatic tumour. The long-term survival was not different for the gallbladder carcinoma patients with and those without nm23 protein expression (data not shown).

Table 4 Comparison of nm23 immunoreactivity between the central and peripheral part of the tumour

nm23 immunoreactivity in central tumour cells	nm23 immunoreactivity in peripheral tumour cells			
	−	+	++	+++
−	23	1	0	0
+	26	7	3	0
++	14	7	3	1
+++	1	3	1	1

Central>Peripheral 52 (58%)
Central=Peripheral 34 (35%)
Central<Peripheral 5 (7%)
p=0.044[a]

[a] See foot note for Table 1. Significant correlation was detected (p=0.044)

Nm23 immunoreactivity in tumour cells invading the surrounding stroma at the border of tumour cell nests was compared with that in cells at the central portion of the tumour. Immunoreactivity of nm23 in the central portion tends to be stronger than that in the peripheral portion (Fig. 2).

Significant correlation was detected upon Kendall Tau b analysis, as shown in Table 4. Fifty-eight percent of the cases showed reduction of nm23 protein expression in tumour cells at the border between the tumour and stroma (peripheral part) compared with the centre, whereas

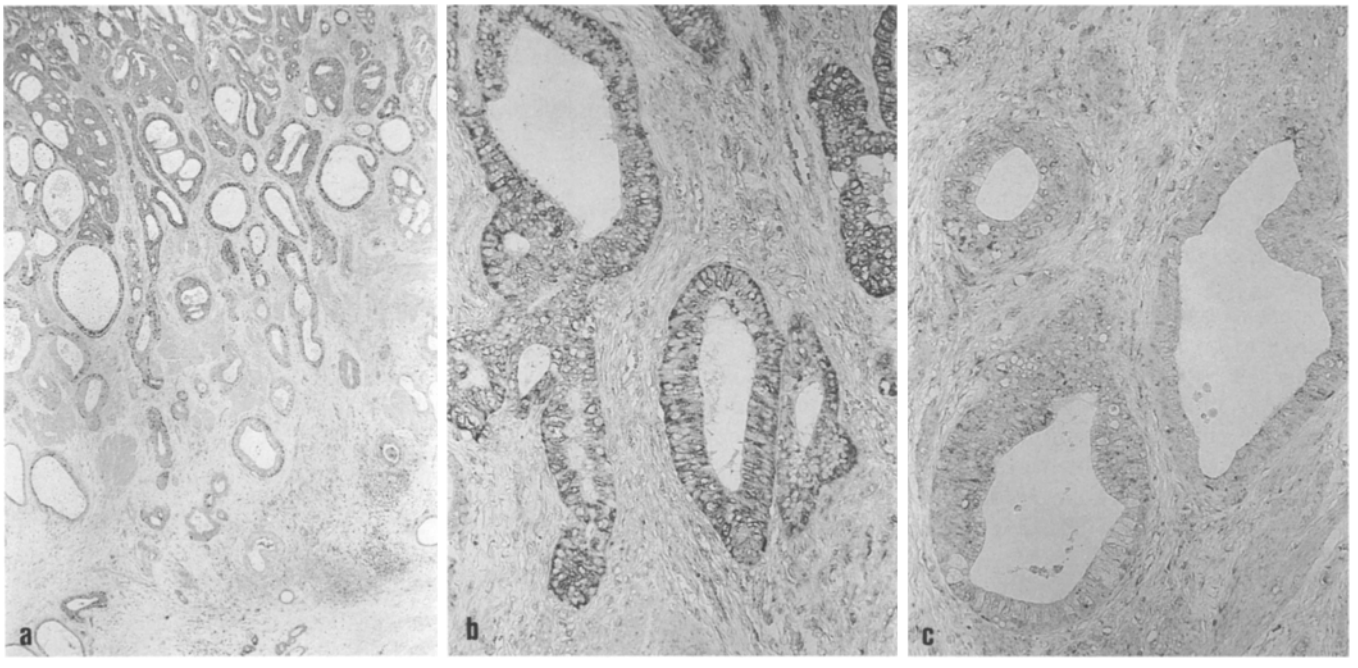

Fig. 2a–c Immunostaining of nm23 protein in a well-differentiated adenocarcinoma. **a** Lower magnification of the tumour, i.e. ×40. **b** Central part of the tumour. Strong staining immunoreactivity to nm23 is observed in most tumour cells at the centre of the tumour. ×400. **c** Peripheral part of the tumour. Tumour cells at the border between the tumour and the stromal are almost negative to nm23 protein. ×400

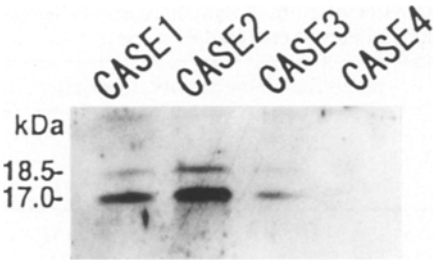

Fig. 3 Western blot analysis of nm23 protein in gallbladder carcinomas. Two bands of 17.0 and 18.5 kDa were detected in cases 1, 2 and 3. Case 1, poorly differentiated adenocarcinoma (T1N1M0, stage III); case 2, moderately differentiated adenocarcinoma (T2N0M0, stage II, the same as Fig. 1b); case 3, well-differentiated adenocarcinoma (T1N0M0, stage I); case 4, well-differentiated adenocarcinoma (T3N0M0, stage III). The grade of nm23 immunostaining in cases 1, 2, 3 and 4 was ++, +++, ++ and -, respectively

only 7% of the cases showed increase in the nm23 protein expression in tumour cells at the border.

To confirm the expression of nm23 protein in the tumour tissue, Western blot analysis using the same antibody was performed in some cases for whom frozen tissues were available. As shown in Fig. 3, two bands of 17.0 kDa and 18.5 kDa were detected in 3 of the 4 gallbladder carcinomas examined. The level of nm23 expression in Western blotting was well correlated with the grade of immunostaining.

Discussion

A candidate suppressor gene for tumour metastasis, nm23 H1 and H2, encodes NDP kinase-A and -B, respectively [3]. It has recently been reported that nm23 H2 protein is identical to c-*myc* transcriptional factor (PuF) [12, 13]. Therefore, it is likely that nm23 may have a role in tumorigenesis through c-*myc* induction in addition to its role in metastasis inhibition. In fact, we have found that gastric and colorectal carcinomas express nm23 at a higher level than corresponding non-neoplastic mucosa [2, 11]. In the present study, more than 70% of gallbladder carcinomas expressed nm23 protein, while non-neoplastic mucosa only occasionally showed weak immunoreactivity to nm23 protein. This finding suggests that nm23 may play a part in the development of gallbladder carcinoma. Nm23/NDP kinase is known to be involved in *ras*-related signal transduction [18]. So far, however, nothing is known about the possible interrelationship between nm23 and the mutated K-*ras* gene which is thought to be involved in tumorigenesis of gallbladder carcinoma [8].

Many reports have emphasized an inverse correlation relationship between nm23 expression and metastatic ability in tumour cells. In breast carcinomas, high levels of nm23 mRNA and protein are associated with low metastatic potential and good prognosis for the patient [5, 7]. We have also reported that the level of nm23 expression is inversely correlated with advanced tumour stage and metastasis of gastric and colorectal carcinomas [2, 15]. However, this may not necessarily be true for all types of carcinoma. Positive correlation between the level of nm23 expression and metastatic ability has been detected in pancreatic carcinomas [10] and neuroblastomas [4], while no correlation was detected in lung adenocarcinoma [6]. In the present study, the expression of nm23 protein was not correlated with metastasis. More than 70% of cases with lymph node metastasis showed nm23 immunoreactivity. Therefore, we concluded that nm23 expression does not have implications for inhibition of metastasis in gallbladder carcinomas. It is likely that the role of nm23 in metastasis differs depending on types of cancer.

We found that many of the cases showed lower nm23 protein expression in tumour cells invading the surrounding stroma at the border of tumour cell nests than in cells at the centre of the tumour. However, we did not detect any correlation between nm23 expression and tumour stage or depth of tumour invasion. Therefore, we cannot at present conclude a significant role of nm23 in local invasion of tumour cells. Furthermore, the mechanism of reduced nm23 expression in the peripheral portion of the tumour is unclear. The invasive properties of tumour cells are modulated by a variety of molecules, such as cadherins, integrins, and hepatocyte growth factor (HGF)/scatter factor. We should clarify the interaction of nm23 with these molecules to get solid evidence of a role for nm23 in tumour invasion.

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References

1. Albores-Saavedra J, Henson DE, Sobin LH (1991) Histological typing of tumors of the gallbladder carcinoma and extrahepatic bile ducts. In: WHO International histological typing of tumours. Springer, Berlin Heidelberg New York
2. Ayhan A, Yasui W, Yokozaki H, Kitadai Y, Tahara E (1993) Reduced expression of nm23 protein is associated with advanced tumor stage and distant metastases in human colorectal carcinomas. Virchows Arch [B] 63: 213–218
3. Gilles AM, Presecan E, Vonica A, Lascu I (1991) Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexiameric enzyme. J Biol Chem 266: 8784–8789
4. Hailat N, Keim DR, Melhem RF, Zhu XX, Eckerskorn C, Brodeur GM, Reynolds CP, Seeger RC, Lottspeich F, Strahler JR, Hanash SM (1991) High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-*myc* gene amplification. J Clin Invest 88: 341–345
5. Hennessy C, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ (1991) Expression of the antimetastatic gene nm23 in human breast cancer: an association with good prognosis. J Natl Cancer Inst 83: 281–285
6. Higashiyama M, Doi O, Yokouchi H, Kodama K, Nakamori S, Tateishi R, Kimura N (1992) Immunohistochemical analysis of nm23 gene product/NDP kinase expression in pulmonary

adenocarcinoma: lack of prognostic value. Br J Cancer 66: 533–536
7. Hirayama R, Sawai S, Takagi Y, Mishima Y, Kimura N, Shimada N, Esaki Y, Kurashima C, Utsunomiya M, Hirokawa K (1991) Positive relationship between expression of anti-metastatic factor (nm23 gene product or nucleoside diphosphate kinase) and good prognosis in human breast cancer. J Natl Cancer Inst 83: 1249–1250
8. Imai M, Hoshi T, Ogawa K (1994) K-*ras* codon 12 mutation in biliary tract tumors detected by polymerase chain reaction denaturating gradient gel electrophoresis. Cancer 73: 2727–2733
9. Kameda T, Yasui W, Yoshida K, Tsujino T, Nakayama H, Itoh M, Tahara E (1990) Expression of ERBB2 in human gastric carcinomas: relationship between $p185^{ERBB2}$ expression and the gene amplification. Cancer Res 50: 8002–8009
10. Nakamori S, Ishikawa O, Ohhigashi H, Kameyama M, Furukawa H, Sasaki Y, Inaji H, Higashiyama M, Imaoka S, Iwanaga T, Kimura N (1993) Expression of nucleoside diphosphate kinase/nm23 gene product in human pancreatic cancer: an association with lymph node metastasis and tumor invasion. Clin Exp Metastasis 11: 151–158
11. Nakayama H, Yasui W, Yokozaki H, Tahara E (1993) Reduced expression of nm23 is associated with metastasis of human gastric carcinoma. Jpn J Cancer Res 84: 184–190
12. Postel EH, Ferrone CA (1994) Nucleotide diphosphate kinase enzyme activity of nm23-H2/PuF is not required for its DNA binding and in vitro transcriptional functions. J Biol Chem 269: 12 8627–8630
13. Postel EH, Berberich SJ, Flint SJ, Ferrone CA (1993) Human c-*myc* transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. Science 261: 478–480
14. Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Baker E, Margulies IMK, King CR, Liotta LA, Steeg PS (1989) Reduced nm23/Awd protein in tumor metastasis and aberrant *Drosophila* development. Nature 342: 177–180
15. Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS (1991) Identification of a second human nm23 gene, nm23-H2. Cancer Res 51: 445–449
16. Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge, JE, Liotta LA, Sobel ME (1988) Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 80: 200–204
17. Takagi S, Naito E, Yamanouchi H, Ohtsuka H, Kominami R, Yamamoto M (1994) Mutation of the p53 gene in gallbladder cancer. Tohoku J Exp Med 172: 283–289
18. Teng SHF, Engele CM, Venkatesh TR (1991) A product of the *prune* locus of *Drosophila* is similar to mammalian GTPase-activating protein. Nature 353: 437–440
19. UICC, International Union Against Cancer (1989) TNM-atlas: illustrated guide to the TNM/pTNM classification of malignant tumors [Spiessl B, Beahrs OH, Hermanek P, Hutter RVP, Scheibe O, Sobin LH, Wagner G (eds)]. Springer, New York Berlin Heidelberg