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IMMUNOHISTOCHEMICAL EXPRESSION OF nm23 GENE PRODUCT CORRELATES WITH LYMPH NODE METASTASIS AND ADVERSE PROGNOSIS IN GALLBLADDER CARCINOMAS INVADING THE SUBSEROSAL LAYER

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Levels of nm23 gene product/NDP kinase expression have been demonstrated to be inversely correlated with metastatic potential in some tumors, but not in others. It is not yet clear, whether the NDP kinase is associated with metastatic potential in gallbladder carcinomas that has invaded the subserosal layer (Gb-ssca). We therefore immunohistochemically examined the expression of NDP kinase-A in Gb-ssca in order to clarify associations between its expression and the clinical features of the disease. Of the 40 patients tested, 25 (63%; Group B) showed strong immunoreactinity for NDP kinase-A in most carcinoma cells within the subserosal tumor tissue, while 15 (37%; Group A) contained few or no NDP kinase-A-positive carcinoma cells. In addition, carcinoma cells in the mucosa showed lower NDP kinase-A immunoreactively than those in the subserosa in 11 cases (27%). In Gb-ssca, high levels of NDP kinase-A expression were associated with positive status of distant lymph node ($p<0.05$) and poorer prognosis ($p<0.05$). These findings are consistent with the view that higher levels of NDP kinase expression are associated with carcinoma progression in Gb-ssca and that it may be a prognosis-regulating factor in Gb-ssca. It is also suggested that expression of NDP kinase-A in Gb-ssca, may not only have implications for metastasis but that it may also play a part in local invasion.

Introduction

Accurate prediction of the malignant potential of gallbladder carcinomas that has invaded the subserosal layer (Gb-ssca), which is reflected in biological features, such as progression, invasiveness and metastasis, is an important goal in clinical oncology. However, no reliable prognostic markers for immunohistochemical application have been reported in Gb-ssca.

A close relationship between the nm23 gene and its product, and the metastatic potential of certain carcinoma cells has recently been recognized[1)~4)]. The expression of nucleoside diphosphate kinase (NDP kinase), which is now known to be identical to the nm23 gene product[3)~7)], has been investigated in breast carcinoma immunohistochemically, and the results suggest that the NDP kinase expression may be a useful prognostic marker[8)]. To our knowledge, no product level studies of the nm23 gene have yet been carried out in Gb-ssca. we therefore performed an immunohistochemical analysis of NDP kinase expression in Gb-ssca to determine its prognostic value.

Materials and Methods

Formalin-fixed, paraffin-embedded tissue blocks from 40 specimens of Gb-ssca surgically resected in our institute were analyzed. Of the 40 patients, 17 were men and 23 were women; their ages ranged from 35 to 81 years (mean: 66). The General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract proposed by the Japanese Society of Biliary Surgery[9] were followed in describing the pathological findings obtained. Tumors of the gallbladder were divided into 3 groups based on difference in histological type: well, histological types of carcinoma were papillary adenocarcinoma and well differentiated type tubular adenocarcinoma; moderate, was moderately differentiated type tubular adenocarcinoma and poor, were poorly differentiated type tubular adenocarcinoma, mucinous adenocarcinoma squamous cell carcinoma and undifferentiated carcinoma.

Immunohistochemical staining was performed on deparaffinized sections at room temperature according to the biotin-streptavidin complex method[10] using a commercial kit (DAKOLSAB Kit, DAKO Japan Co) and a mouse monoclonal antibody of nm23/NDP kinase-A protein (antigen used for immunizations was NDP kinase-A purified from human erythrocytes, Novocastra Laboratories Ltd.)[11]. Briefly, the sections (4 μm thick) were treated for 10 minutes with 0.3% H_2O_2-methanol to block endogenous peroxidase. After washing with phosphate-buffered saline (PBS, pH 7.4) three times, the sections were incubated for 1 hour with mouse monoclonal antibody (working dilution=1 : 100 or normal mouse IgG (working

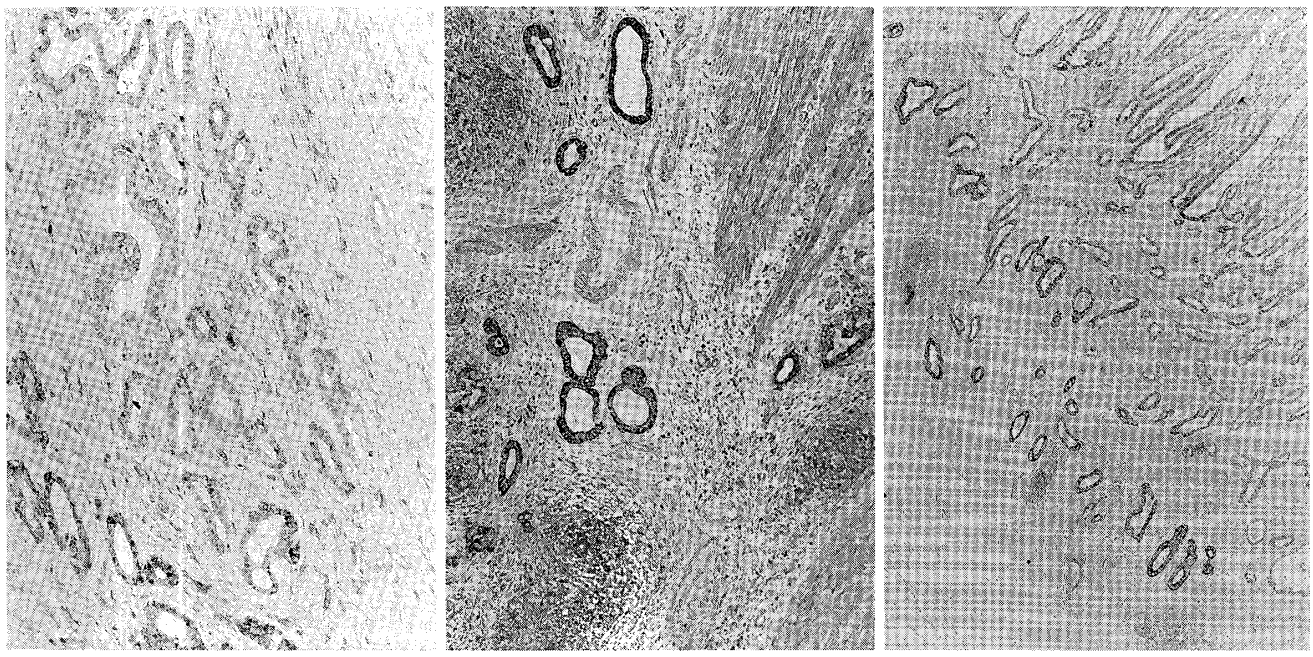

Fig. 1 nm23 gene product/NDP kinase-A expression in gallbladder cancer invading to subserosal layer by immunostaining using anti-NDP kinase-A antibody

a Tissue from Group A patients shows immunoreactivity for NDP kinase-A in a few cancer cells.

b In tissue from a Group B patients, NDP kinase-A is strongly expressed in almost all the cancer cells.

c In tissue from another Group B patient, tumor cells in the mucosa and the lamina muscular is show reduced NDP kinase-A expression in comparison with those in the subserosa.

dilution=1 : 100), as a negative control. After repeated washings with PBS, the sections were reacted for 20 minutes with biotinylated goat anti-mouse IgG and then washed in PBS. The sections were further incubated for 20 minutes with peroxidase-conjugated streptavidin followed by washing in three changes of PBS. The reaction product was developed by incubation for 5 minutes in 0.61 M Tris-HCl buffer (pH 7.4) containing 0.05% 3,3′-diaminobenzidine-4 HCl (Sigma Chemical Co., St. Louis, Mo.) and 0.01% H_2O_2.

Immunostaining results were assessed semi-quantitatively by two of the authors, taking into account the percentage of NDP kinase-positive carcinoma cells within maximum cut-surface specimens of subserosal tumor tissue. The patients were classified into two groups dependent on the percent of NDP kinase-positive carcinoma cells that invading the subserosal layer: patients with less than 50% NDP kinase-positive carcinoma cells (Group A), and those with 50% or more NDP kinase-positive carcinoma cells (Group B). The chi-square test was used for statistical analysis. The Kaplan-Meier method was used to calculate postoperative survival rate, and prognostic significance was evaluated by the generalized Wilcoxon test.

Results

NDP kinase-A expression was observed in the cytoplasm of Gb-ssca cell in this immunohistochemical study using formalin-fixed and paraffin-embedded samples. The noncancerous part of the gallbladder contained little or no NDP kinase, and 27% (11 cases) showed reduction of nm23 immunoreactively in carcinoma cells invading the mucosa including the lamina muscularis compared with carcinoma cells in the subserosa (Fig. 1). The other cases (29 cases, 73%) showed homogeneous staining pattern in carcinoma cells invading the mucosa and subserosa.

There were 15 cases (37%) in Group A and 25 cases (63%) in Group B. Table 1 and Table 2 show the relationship between NDP kinase immuno-reactivity and clinicopathological sta-

Table 1 Correlation between NDP kinase-A expression and clinicopathological factors

	No. of patients	Group A(%)	Group B(%)
hinf			
(−)	15	8(53)	7(47)
(+)	25	7(28)	18(72)
binf			
(−)	32	13(41)	19(59)
(+)	8	2(25)	6(75)
liver metastasis			
(−)	36	15(42)	21(58)
(+)	4	0(0)	4(100)
LN metastasis*			
0～1	27	13(48)	14(52)
2～	13	2(15)	11(85)
		**	
ly involvement*			
0～1	19	8(43)	11(57)
2～	21	7(33)	14(67)
v involvement*			
0～1	23	11(48)	12(52)
2～	17	4(23)	13(77)
Stage			
I, II	24	11(46)	13(54)
III, IV	16	4(25)	12(75)

* : on the basis of General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract (3rd ed, 1993),

** : $p<0.05$, hinf : hepatic infiltration, binf : infiltration of the hepatoduodenal ligament, LN : lymph nodal, ly : lymphatic, v : vein.

As for the definition of groups A and B, see text.

Table 2 Correlation between histological type and growth pattern, and NDP kinase-A expression

	No. of patients	Histology			
		good	moderate	poor	
Group A	15	9(60)	3(20)	3(20)	(%)
Group B	25	13(52)	7(28)	5(20)	

	No. of patients	Growth pattern			
		α	β	γ	
Group A	15	2(13)	7(47)	6(40)	(%)
Group B	25	2(8)	12(48)	11(44)	

α : expansive growth, β : intermediate between α and γ, γ : infiltrative growth.

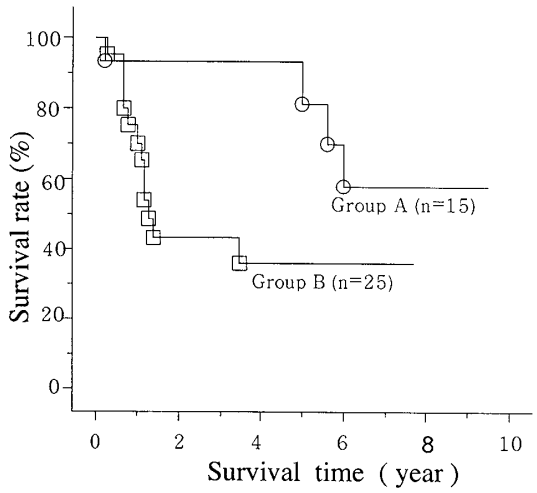

Fig.2 Kaplan-Meier survival curve of patients with gallbladder cancer invading subserosal layer, with regard to NDP kinase-A expression The Group B had a poor survival rate than Group A ($p<0.05$; generalized Wilcoxon test).

tus. NDP kinase was expressed independently of pathologic stage, hepatic infiltration (hinf), infiltration of the hepatoduodenal ligament (binf), angiolymphatic invasion, growth pattern, liver metastasis and histological differentiation. The incidence of distant regional lymph node metastasis was significantly higher in Group B (85%) than in Group A (15%) ($p<0.05$). The trends towards being positive for invasion of the liver or the hepatoduodenal ligament, being positive for angiolymphatic invasion, liver metastasis, and advanced pathological stage in Group B were not statistically significant.

Postoperative survival curves for the two groups, including patients in stages I, II, III and IV who underwent curative operations, are shown in Fig. 2. The average 5-year survival rate was 82% in Group A and 36% in Group B ($p<0.05$), and the difference in survival rates between the two groups was significant.

Discussion

The nm23-H1 gene was originally identified as a metastasis suppressor gene[1], and it has been found to be inversely associated with metastatic potential in human breast carcinomas[8)12)13)] and melanoma[14]. Whether the nm23 gene product is associated with metastatic potential in Gb-ssca is not yet clear. We immunohistochemically investigated for the expression of NDP kinase-A in Gb-ssca in order to clarify the association of its expression with the clinical features of the disease.

In the present study in which used an immunohistochemical technique, we demonstrated that high NDP kinase-A levels in Gb-ssca were associated with positive status of distant lymph node and poor prognosis. The results for Gb-ssca conflict with the previous reports that NDP kinase expression is inversely associated with metastatic potential[1)8)12)~14)]. However, results similar to our own have been noted in regard to several other human malignancies. Aggressive neuroblastoma showed higher levels of NDP kinase with N-*myc* gene amplification[15] and in colonic carcinomas, increased NDP kinase expression has been found in neoplastic colon tissue compared with the levels in normal mucosa from the same individuals[16]. Our present data confirm that NDP kinase expression is associated with indicators of carcinoma malignancy, such as metastatic potential and prognosis in Gb-ssca.

The reasons for these discrepancies regarding the significance of NDP kinase expression in human malignancy are not understood, but two possibilities can be considered. First, the biological significance of NDP kinase may differ considerably in different tissues. Second, the NDP kinase has now been demonstrated to consist of two isotypes, nm23-H1 and nm23-H2, which are identical to chain A and B of NDP kinase, respectively but it is not known if nm23-H1 and nm23-H2, if proven to be NDP kinase, participate in different functions[17)18)]. The expression of the nm23-H1 isotype is reduced in breast cancer with higher metastatic potential[13)17)19)] and allelic loss of the nm23-H1 gene on chromosome 17 is observed in pulmonary adenocarcinoma[19], but it is still unknown which type is predominant with regard to the metastatic potential of Gb-ssca. In this present study, the expression of NDP kinase-A was

positively associated with distant lymph node metastasis and poor prognosis.

NDP kinase provides intracellular pools of nucleoside triphosphate, regulating polymerization of microtubules in the mitotic spindle and cytoskeleton, and supplies GTP to G-protein in signal transduction[5)20)]. The predicted amino acid sequence of NDP kinase suggests that NDP kinase acts as a transcription factor[21)22)]. The possibility remains that NDP kinase that generats GTP, may play a variety of roles, depending on other factors, which vary from cell to cell and from tumor to tumor. We have shown that immunohistochemical staining of NDP kinase-A is positively associated with distant lymph node metastasis and poor prognosis in Gb-ssca. These findings suggest that NDP kinase-A may play an important role in cancer progression or aggressiveness by altering its expression in a tissue-specific manner and that NDP kinase-A expression might be useful as a prognostic indicator in Gb-ssca. Furthermore, results show the low NDP kinase-A immunoreactivity (Group A) had significantly higher 5 years survival rate than those with high NDP kinase-A immunoreactivity (Group B), but the six years significantly higher survival rate cannot be found and the data show the decrease in survival rate of Group B occurred within the first two years (Fig. 2). The expression of NDP kinase-A may be serve as a time-dependent prognostic indicator. On the other hand, 27% of the cases showed lower NDP kinase-A immunoreactively in carcinoma cells invading the mucosa and the lamina muscularis than in carcinoma cell at the subserosa. It is possible that expression of NDP kinase-A in Gb-ssca may not only have implication for metastasis but may also play a part in local invasion.

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漿膜下層浸潤胆嚢癌における nm23 遺伝子翻訳産物の免疫組織化学的発現率とリンパ節転移および予後との関連

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乳癌ほかいくつかの癌で癌組織内の nm23遺伝子翻訳産物/NDP kinase の発現の多寡が転移および予後に深く関与していることが明らかにされ，予後因子としての有用性が期待されている．しかし漿膜下層浸潤胆嚢癌における検討は文献的にはまだ認められない．今回われわれは胆嚢癌手術材料について免疫組織化学的方法を用い，NDP kinase-A と漿膜下層浸潤胆嚢癌との関連を検討した．漿膜下層浸潤胆嚢癌40例を対象とし癌細胞の NDP kinase-A 発現50%未満（A 群）と50%以上（B 群）に分けて検討すると，2 群以上の遠位リンパ節転移率は B 群に有意に高く，予後も有意に不良であった．漿膜下層浸潤胆嚢癌においては NDP kinase-A 発現はリンパ節転移および予後とは有意な関連があり，転移予後の予測因子として有用性が示唆された．また11症例（27%）では粘膜内癌，筋層浸潤癌の部より漿膜下層浸潤癌の部において NDP kinase-A 染色程度は強く認められ，NDP kinase-A は胆嚢癌の進展に関連することが示唆された．