The Role of *nm23-H1* in the Progression of Transitional Cell Bladder Cancer[1]

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ABSTRACT

The *nm23* gene was initially cloned as a metastasis suppressor gene, but the clinical relevance of *nm23-H1* as a metastasis suppressor or prognostic indicator for human cancers remains enigmatic. Given that gene expression is regulated at the tissue-specific level, we studied the molecular mechanisms of *nm23-H1* expression in human bladder cancer cell lines and the clinical importance of protein product (NM23-H1) in association with patient outcome ($n = 257$) by immunohistochemistry. We demonstrated that *nm23-H1* is expressed in bladder cancer cells without genomic alterations. High NM23-H1 expression was found in 39 cases (15.2%), intermediate expression in 119 cases (46.3%), and low NM23-H1 in 99 cases (38.5%). NM23-H1 was inversely related to staging classification or tumor size ($P < 0.05$), with the most significant difference being observed between pT_a tumors and those of pT_1-pT_3 bladder cancer ($P = 0.01$). Reduced NM23-H1, defined as intermediate and low levels of expression, tended to have a higher risk of tumor metastasis ($P = 0.06$) or poor longtime survival ($P = 0.07$). In the subset of grade 2 bladder tumors, reduced NM23-H1 significantly correlated with the occurrence of tumor metastasis or poor patient survival ($P < 0.05$). These findings overall suggest that *nm23-H1* may play an important role in suppressing the early step of carcinogenesis and thus act as an invasion suppressor for human bladder cancer. A prospective study is required to clarify the potential of the molecular marker in prediction of disease progression.

INTRODUCTION

The *nm23-H1* gene (*NME1*), localized on chromosome 17q21.3, was first isolated as a metastasis suppressor gene by differential screening of cDNA library from low and high metastatic clones of a murine melanoma cell line (1). Presently, a total of five *nm23* family members have been identified, *i.e.*, *nm23-H1*, *nm23-H2*, *DR-nm23*, *nm23-H4*, and *nm23-H5* (2–6). *nm23-H2* was identified as coding for the B subunit of NDP[3] kinase, as compared with the A subunit coded for by *nm23-H1*. *DR-nm23* is highly expressed in the myeloid leukemia (4), and *nm23-H4* presents characteristics of a mitochondrial enzyme (5). The expression of *nm23-H5* is reported to be specific to the testis germ cells (6). Taken together, the gene products of this family are thought to function in the growth, development, differentiation, and apoptosis of normal tissue.

The clinical relevance of *nm23-H1* as a metastasis suppressor for human cancers remains enigmatic. Reduced *nm23-H1* expression was reported to correlate with tumor metastasis in the carcinomas of liver (7–9), breast (10), stomach (11), and ovary (12–14). Transfection study of highly metastatic human breast cancer cell line essentially supports this hypothesis in the context of metastatic potential *in vivo*, as well as colonization and cell motility *in vitro* (15). However, other groups could not verify the significance of *nm23-H1* in terms of tumor invasion (11, 16–18) or metastasis (8, 10, 16, 17, 19–24). As for prognostic value, expression of the protein product of *nm23-H1* (NM23-H1) was shown to inversely relate to patient survival in the carcinomas of stomach (11), larynx (25), and ovary (14), but not in the carcinomas of breast (21), lung (22), uterine cervix (24), and kidney (26). On the contrary, overexpression of NM23-H1, without allelic deletion or genetic amplification, was positively associated with progression of cancers of the thyroid (23), pancreas (27), and lung (28). The disparities described above suggest that *nm23-H1* may have a distinct, and possibly opposite, role in tumors of different origin.

Literature reports for *nm23-H1* in bladder cancer are also contradictory (29–33). NM23-H1 was demonstrated to inversely correlate with tumor staging, histological differentiation, or clinical outcome (29, 31–33). However, there are reports showing a positive relationship to histological grading (30, 32), muscular invasion (30, 31), or proliferating cell nuclear antigen expression (30, 32), implying a positive growth-regulatory role for *nm23-H1* in bladder tumorigenesis. To clarify this issue, we performed this comprehensive cohort study. The molecular mechanisms of *nm23-H1* expression in bladder cancer cell lines were investigated in the context of genomic structure, zygosity status, and mRNA level. A clinical cohort was examined for

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[3] The abbreviations used are: NDP, nucleoside diphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGF, epidermal growth factor.

significance of *nm23-H1* in the progression of bladder carcinogenesis, and for its prognostic value compared with conventional biological indicators.

MATERIALS AND METHODS

Cell Lines and Culture. Human bladder cancer cell lines J82, RT4, T24, and SCaBER were obtained from the American Type Culture Collection (Manassas, VA). The TSGH-8301 cell line was derived from a grade 2 papillary bladder cancer (34). Cells were maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY) 2 mM L-glutamine, and 110 mg/liter sodium pyruvate at 37°C in a 5% CO_2-humidified atmosphere.

Northern Blot Hybridization Study. RNA preparation and Northern blotting were performed as previously described in detail (35). Briefly, total RNA (20 μg) from exponentially growing cells were resolved by 1% agarose gel electrophoresis, and transferred to GeneScreen nylon membranes (New England Nuclear Nuclides & Sources Division, Boston, MA). After overnight prehybridization, the membranes were hybridized with *ras*, *nm23-H1*, *nm23-H2*, or GAPDH cDNA probe labeled with [α-^{32} P]dCTP by using random primer labeling kit (Life Technologies, Inc.). The resulting signals of *ras*, *nm23-H1*, *nm23-H2*, and GAPDH were evaluated by a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Expression of *nm23-H1* in Relation to Cell Proliferation. To determine the relationship between *nm23-H1* and cell proliferation, J82 cells containing high levels of receptor for EGF (36) and TSGH-8301 cells with low levels of receptor were analyzed for *nm23-H1* mRNA alteration after stimulation with EGF.

Reverse Transcription-PCR Amplification and Sequencing. A mutational screening of the coding region of the *nm23-H1* gene was studied on all cancer cell lines as previously described (37). The primers for PCR were hnm23c 5′-AAGAATTCTCGGGTCGAGGCCGCCATG and hnm23 3′-GGGAATTCTGCGCCAGGGAGGATACAG. The PCR products separated by 2.0% agarose gel (QIAGEN Inc., Santa Clarita, CA) were purified and digested with *Eco*RI (Boehringer Mannheim, Indianapolis, IN). Then, inserts were cloned into pCR^{R}2.1 vector (Invitrogen Inc., San Diego, CA). Direct sequencing of the plasmids with *nm23-H1* insert was performed by Sanger's dideoxynucleotide chain-termination method with the Sequenase Version 2.0 DNA sequencing kit (Amersham, Buckinghamshire, United Kingdom). A minimum of three individual clones from each PCR was analyzed to confirm the sequence.

Analysis of Microsatellite Repeats. A CA repeat at the *nm23-H1* loci was amplified by PCR as described previously (38). The primer sets were 5′-TTGACCGGGGTAGAGAACTC and 5′-TCTCAGTACTTCCCGTGACC. The PCR products were separated by electrophoresis in 6% acrylamide sequencing gels. Because no constitutional DNA was available for loss of heterozygosity assessment, those with two distinct PCR products were classified as heterozygous. If only one expected PCR product was observed, the cell lines were interpreted as hemizygous or homozygous within genomic DNA.

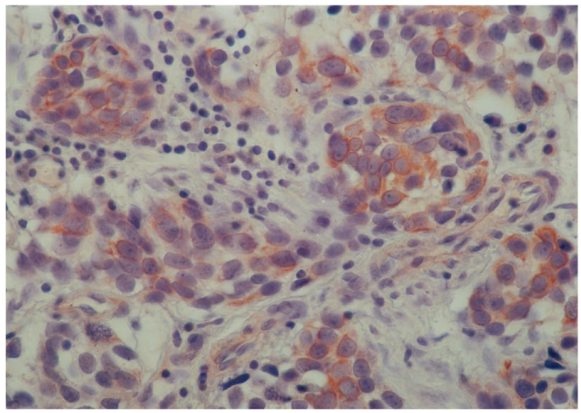

Fig. 1 Immunohistochemical analysis for NM23-H1 expression in human bladder cancer cells. Most of the tumor cells showed strong cytoplasmic staining for NM23-H1, whereas the infiltrating lymphocytes and endothelium of capillary were all nonreactive (original magnification, ×300).

Clinicopathological Characteristics. In this cohort, patients treated in the National Cheng Kung University Hospital were collected ($n = 257$). There were 165 males and 90 female patients, ranging in age from 29 to 90 years of age (63 ± 11 years old). All cases were reviewed for histological grading according to the WHO classification (1973). Clinical staging was determined according to the tumor-node-metastasis staging protocol of the American Joint Committee on Cancer (1983) with survey of the clinical details, image studies, and pathological data. The survival status was determined by outpatient clinic record and/or confirmed by interview with their families. Clinical follow-up ranged from 24 months to 95 months (median, 54 months).

Immunohistochemical Analysis of NM23-H1 Expression. Appropriate sections were cut for immunostaining. The monoclonal anti-*nm23-H1* antibody (Santa Cruz, CA) raised against cytoplasmic domain of human NM23-H1 protein was used as the primary antibody and incubated with sections for 2 h at room temperature. The optimal dilution (1:50) was determined by using human colonic mucosae as the control, in which nonmucinous, colonic surface epithelium is usually positive for NM23-H1 (39). The StrAviGen Super Sensitive MultiLink kit (BioGenex, San Ramon, CA) was used to detect the resulting immune complex. The procedures of blocking, linking, and labeling of binding reaction were carried out as manufacturer's instructions. Peroxidase activity was visualized by using the aminoethyl carbazole substrate kit (Zymed Laboratory, Inc., San Francisco, CA). Sections were finally counterstained with hematoxylin. Negative control was performed by incubation of nonimmune mouse IgG in substitution for primary antibody.

When evaluating the expression of NM23-H1, we used the scoring criteria described previously in the literature (24). Tissue sections exhibiting immunostaining in >70% of tumor cells were classified as "high expression" (Fig. 1), whereas those with <70% reactivity were classified as "intermediate expression." Those revealed to have <5% of NM23-H1 staining or no any immunoreactivity were classified as "low expression."

Table 1 Correlation of NM-23H1 with clinicopathological factors of bladder cancer

Factors	Levels of NM-23H1 expression			
	High	Intermediate	Low	*P*
Grade				
1	7	17	24	NS[a]
2	18	58	42	
3	14	44	33	
Stage				
pT_a	25	45	46	0.04[b]
pT_1–T_3	14	69	47	
N(+), M(+)	0	6	6	
Size (cm)				
≤1	13	23	32	NS[c]
1 ≪3	15	51	40	
≥3	11	45	27	
Shape				
Papillary	34	95	85	NS
Nodular	5	24	14	
Number				
Single	20	66	55	NS
Multiple	19	53	44	

[a] NS, not significant.

[b] Significant difference between pT_a tumors and pT_1–pT_3 tumors ($P = 0.01$).

[c] Significant difference between tumors of ≤1 cm and those >1 cm (0.04).

Statistics. Correlation between NM23-H1 and clinicopathological indicators of bladder cancer was examined, where suitable, by ANOVA, Fisher's exact test, or χ^2 test. Then, the relations between NM23-H1 expression levels and clinical parameters in question were analyzed by logistic regression. Total survival of patients was calculated by Kaplan-Meier analysis, and the significance of NM23-H1 in relation to tumor recurrence, metastasis, or patient survival was assessed by means of a Cochran-Mantel-Haenszel test (log-rank test). Only those variables with a $P < 0.05$ were considered significant.

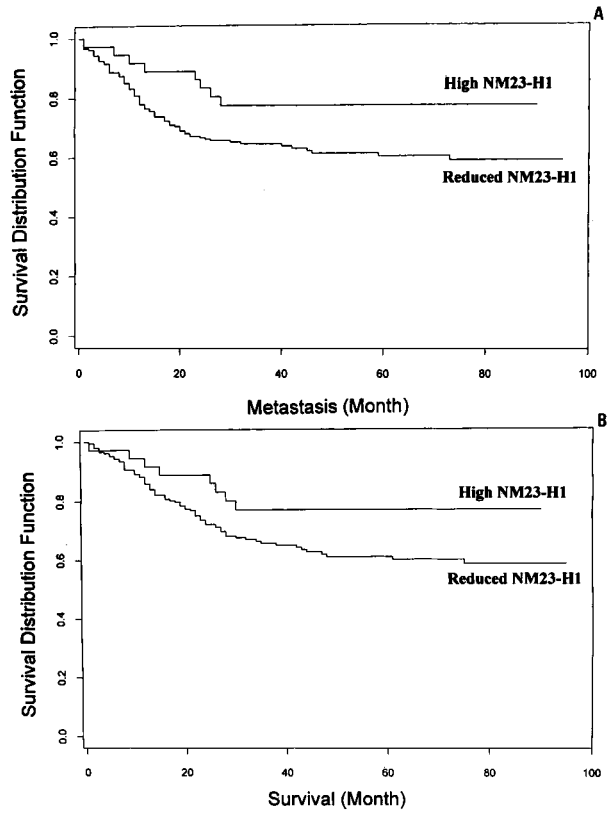

Fig. 2 Kaplan-Meier analysis of NM23-H1 expression in relation to clinical outcome. Overall survival of patients was estimated by using the Kaplan-Meier method, and the significance of NM23-H1 in association with tumor metastasis or patient survival was assessed by means of the log-rank test. *A*, tumors with high expression of NM23-H1 tended to have a lower metastatic potential ($P = 0.06$). *B*, patients with reduced NM23-H1, defined as low and intermediate levels of expression, tended to have a poor longtime survival ($P = 0.07$).

RESULTS

nm23-H1 mRNA was expressed in all bladder cancer cell lines with *nm23-H1*:GAPDH ratios varied from 0.302 to 1.355 (data not shown). Analysis of *nm23-H1* dinucleotide repeat revealed hemizygous/homozygous for RT4 and T24 cells, whereas the remaining three were heterozygous. Mutational screening of the coding region of *nm23-H1* did not find any point mutation in all of the cancer cells. Taken together, the lack of correlation of mRNA expression with zygosity status and the absence of point mutations in the coding region imply that *nm23-H1* is expressed in bladder cancer cells without genomic alterations. In contrast, *nm23-H2* mRNA was present in all cell lines tested without conspicuous difference (data not shown). Thus, *nm23-H2* was not analyzed in the following study.

Both J82 and TSGH8301 demonstrated a dose-dependent cell proliferation and up-regulation of *ras* mRNA as early as 5 min after treatment with EGF (10 ng/ml). But levels of *nm23-H1* mRNA remained unaltered for up to 3 h (data not shown).

Expression of NM23-H1 in normal urothelium was examined in six cases of nonneoplastic bladder tissue with inflammatory disease. There was weak cytoplasmic staining in the superficial cells of mucosa, comparable with normal control. The nerve fibers and muscle layer of small arteries were also positive for NM23-H1 staining. These elements thus served as an internal control for immunohistochemical evaluation. For bladder cancer ($n = 257$), the expression of NM23-H1 correlated with clinicopathological factors of patients as follows (summarized in Table 1): 39 cases (15.2%) showed high NM23-H1 expression, 119 cases (46.3%) showed intermediate expression, and 99 cases (38.5%) showed low NM23-H1. NM23-H1 expression was inversely related to tumor staging ($P = 0.04$). The greatest difference of expression was observed between tumors of pT_a and those higher than pT_1 ($P = 0.01$). Further, higher NM23-H1 was usually found in the tumors <1 cm ($P = 0.05$). Otherwise, expression of NM23-H1 did not correlate with the remaining biological indicator ($P > 0.1$). Multivariate analysis using the logistic regression model confirmed that tumor stage had the strongest (negative) correlation with NM23-H1 ($P = 0.006$; data not shown).

Then prognostic value of NM23-H1 was tested in the bladder cancer cohort. Reduced NM23-H1 expression, defined as low and intermediate levels of expression, tended to have a

Table 2 Prognostic significance of clinicopathological indicators and NM-23H1 in bladder cancer (by logistic regression model)

	Univariate		Multivariate	
Analysis factor	Recurrence	Survival	Recurrence	Survival
Age	0.06	0.001[b]	0.23	0.005[b]
Sex	0.19	0.39	0.39	0.64
Grade	0.05[a]	0.02[a]	0.82	0.35
Stage	0.001[b]	0.0001[c]	0.02[a]	0.01[a]
Size	0.04[a]	0.004[b]	0.56	0.97
Shape	0.30	0.03[a]	0.30	0.23
Multiplicity	0.0001[c]	0.0004[c]	0.0001[c]	0.002[b]
NM-23-H1	0.54	0.07	0.28	0.13

[a] $P \leq 0.05$.
[b] $P \leq 0.005$.
[c] $P \leq 0.0005$.

higher risk of metastatic potential ($P = 0.06$) and poor patient survival ($P = 0.07$; Fig. 2). But, there was no relationship with tumor recurrence ($P = 0.50$; data not shown). Multivariate analysis did not show NM23-H1 to be a significant factor in predicting tumor recurrence or patient survival ($P > 0.1$) compared with patient age, tumor staging, and multiplicity (Table 2). In the subset of grade 2 bladder cancer, however, reduced NM23-H1 was significantly associated with tumor metastasis ($P = 0.03$) or patient survival ($P = 0.05$; Table 3).

DISCUSSION

In this study, we found that reduced NM23-H1 is related to larger tumors (>1 cm) and higher cancer stages (>stage pT_1), and that NM23-H1 tends to inversely correlate with tumor metastasis or poor clinical outcome. Taken together, *nm23-H1* appears to be a tumor suppressor for bladder cancer. The conclusion is essentially consistent with multiple cohorts of tumors of the breast, liver, stomach, ovary, and colon (7–9, 11–14, 40), but is in sharp contrast to reports from previous bladder cancer cohorts showing a positive association of NM23-H1 with histological grading (30, 32) or muscular invasion (30, 31). The discordance may be partly explained by differential specificity of the antibodies applied in the earlier reports (30, 31, 33). In this investigation, monoclonal antibody reactive to purified NM23-H1 of human origin was used, whereas polyclonal NM23-H1 antibody (30, 33) and monoclonal anti-NDP kinase antibody (31) were used in prior studies. With respect to NDP kinase, enzyme activity has been proved to be unrelated to tumor suppression (41).

The most interesting finding of this investigation, however, is the significant difference of NM23-H1 between papillary noninvasive tumors (stage pT_a) and those with stromal or muscular invasion (higher than stage pT_1), suggesting an invasion-suppressing role for *nm23-H1*. The involvement of *nm23-H1* in suppressing the early step of bladder carcinogenesis is in sharp contrast to the metastasis-suppressing effect proposed in the literature (7–9, 11–14). Moreover, the ability of NM23-H1 in predicting the development of tumor metastasis or survival rate in the subset of grade 2 tumors suggests that *nm23-H1* may be a potential molecular marker for disease progression. A prospective study is under way to test our hypothesis.

As with Liebert *et al.* (42), we found that NM23-H1 is present in the superficial layer of normal urothelium. Similar topographic distribution of NM23-H1 in relation to cellular differentiation was observed in the nonmucinous, colonic surface epithelium (39) and luminal cells of human prostatic gland (43). In developing mice, increased expression of NM23-H1 was associated with functional differentiation in the liver, kidney, skin, intestine, and stomach (44). The lack of alteration of mRNA levels in response to EGF stimulation in the present study supports that expression of *nm23-H1* is independent of cellular proliferation. Nevertheless, the mechanisms by which down-regulation of *nm23-H1* is involved in the progression of bladder cancer remain to be elucidated.

In summary, the findings of this study suggest that *nm23-H1* may play an important role in suppressing the early step of tumorigenesis of human bladder. A prospective longtime study is required to clarify the potential of *nm23-H1* as a molecular marker for disease progression.

Table 3 Prognostic significance of NM23-H1 and biological indicators in grade 2 transitional cell carcinoma of the urinary bladder

Analysis factor	Recurrence	Metastasis	Survival
Stage	0.48	0.04[a]	0.02[a]
Size	0.16	0.38	0.74
Shape	0.61	0.75	0.76
Multiplicity	0.001[b]	0.09	0.01[a]
NM-23-H1	0.26	0.03[a]	0.05[a]

[a] $P \leq 0.05$.
[b] $P \leq 0.005$.

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