The Role of nm23-H1 in the Progression of Transitional Cell Bladder Cancer

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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 most significant difference being observed between pT4a tumors and those of pT1-pT3 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 longterm 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 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, 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...
significant of nm23-H1 in the progress 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% CO2-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 Nucleides & Sources Division, Boston, MA). After overnight prehybridization, the membranes were hybridized with ras, nm23-H1, nm23-H2, or GAPDH cDNA probe labeled with [α-32P]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'-GAATTCTCGGGTCGAGGCCGCCATG and hnm23 3'-AA-AGAATTTCTCAGGAGCCGCACATG and hnm23 3'-GG-GAATTCTGCGCCAGGGAGGATACAG. The PCR products separated by 2.0% agarose gel (QIAGEN Inc., Santa Clarita, CA) were purified and digested with EcoRI and BamHI. 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 homozygous or homozygous within genomic DNA.

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 mucosa 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.”
Statistics. Correlation between NM23-H1 and clinicopathological indicators of bladder cancer was examined, where suitable, by ANOVA, Fisher’s exact test, or \( \chi^2 \) 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.

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 pTa and those higher than pT1 (\( P = 0.01 \)). Further, higher NM23-H1 was usually found in the tumors, \( \geq 1 \) 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 lower metastatic potential (\( P = 0.06 \)). Patients with reduced NM23-H1, defined as low and intermediate levels of expression, tended to have a poor longtime survival (\( P = 0.07 \)).

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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels of NM-23H1 expression</th>
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<tr>
<td></td>
<td>High</td>
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<td>Grade 1</td>
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<td>6</td>
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<tr>
<td>Size (cm)</td>
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* NS, not significant.
# Significant difference between pT a tumors and pT1–pT3 tumors (\( P = 0.01 \)).
* Significant difference between tumors of \( \leq 1 \) cm and those \( > 1 \) cm (0.04).

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 (\( P = 0.07 \)).
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 pT1), 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 pT0) and those with stromal or muscular invasion (higher than stage pT1), 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.

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