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NM23 as a prognostic biomarker in ovarian serous carcinoma

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The *nm23* gene is a reported metastasis suppressor gene. Recent studies have shown that its expression has tissue specificity. The role of *nm23* in human ovarian cancer is still controversial. This study examines the prognostic significance of *nm23* expression in patients with serous ovarian carcinoma. Following comparative proteomics in 13 fresh frozen ovarian serous cancer tissues with other histological types of ovarian cancers, validation was performed using immunohistochemistry on clinically well-designed 73 ovarian serous carcinoma microarray samples that were retrieved from ovarian cancer patients from 1990 to 2003. Statistical analysis of the results was performed using χ^2 test, Cox proportional regression, the Kaplan–Meier method and log-rank test. We found that the expression of *nm23* inversely correlated with peritoneal seeding ($P=0.009$). However, strong *nm23* expression was associated with mortality in patients with ovarian carcinoma in univariate analysis ($P=0.04$). Poor prognostic factors of disease-free survival included tumor residue more than 2 cm ($P=0.02$), bilaterality ($P=0.01$) and peritoneal seeding ($P<0.01$), whereas poor prognostic factors affecting overall survival included peritoneal seeding ($P=0.05$). In Kaplan–Meier analysis, strong *nm23* immunoreactivity correlates with poor overall survival ($P=0.04$) but not with poor disease-free survival. In conclusion, overexpression of *nm23* is independently associated with decreased overall survival in patients with ovarian carcinoma and also significantly correlates with mortality. *Nm23* may have a biological function that leads to poor clinical outcomes in ovarian carcinoma.
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The lethality of serous ovarian carcinomas is primarily attributed to its advanced stage at the time of initial diagnosis. It spreads rapidly along the peritoneum and omentum in approximately 70% of patients and eventually metastasizes.[1] Despite advances in cytotoxic therapies, only 30% of ovarian cancer patients survive 5 years after their initial diagnosis.[1–3] Therefore, understanding the molecular mechanisms regulating the invasion or metastatic behavior of ovarian cancer will likely have a significant impact on the outcomes of this devastating disease and/or on the diagnosis of early-stage ovarian cancer in patients who are at risk for subsequent metastatic progression or relapse.

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In general, cancer metastasis is an active process involving multiple, sequential cellular events that are regulated by numerous genes. In contrast, ovarian carcinoma metastasis is the result of a relatively passive process.[2] Once a tumor disrupts the ovarian capsule, it may grow so large that it extends to and makes contact with the omentum. Alternatively, cells from the primary tumor may be transported to the omentum via the peritoneal fluid.[2] Previous genomic studies have shown that there are little differences in gene expression profiles between ovarian carcinomas and their omentum metastases.[3]

We have previously used a comparative proteomic approach and various other subtractive and comparative methods to examine the gene expression profiles of ovarian carcinomas.[4] The results of these studies have led to the identification of several putative biomarkers for ovarian carcinomas. These candidate genes included *nm23, annexin I, protein phosphatase-1, ferritin, proteasome α-6, N-acetyl glucosamine kinase (NAGK) and an A6-related*

protein.[4] Interestingly, we found that the *nm23/NDP* kinase family is typically overexpressed in ovarian cancers but generally underexpressed in metastatic cells. In the current study, we use proteomics and immunohistochemistry to examine the biological role of nm23 in serous ovarian carcinomas.

Materials and methods

Tissue Specimens and Sample Preparation for 2E

Fresh frozen samples of 13 ovarian serous cancer tissues were obtained from the Yonsei University College of Medicine (Table 1). Fresh frozen tissues were prepared from eight other types of ovarian cancers (five endometrioid cancers and three mucinous cancers) and three samples of kidney, liver, lung and cervical cancer tissues. All samples were prepared with the consent of the patients who donated tissues.

Table 1 Correlation of nm23 expression and clinical variables

Variables	*nm23*	P-*value*
Age (years)		0.51
<50	42	
≥50	35	
Stage		0.06
I, II	53	
III, IV	24	
Grade		0.12
1–2	30	
3	47	
Ascites		0.97
Negative	40	
Positive	37	
CA125 level (U/ml)		0.76
<35	13	
35–100	23	
>100	41	
Residual volume		0.40
Optimal	43	
Non-optimal	34	
Ovarian bilateralness		0.80
Unilateral	40	
Bilateral	37	
Peritoneal seeding		0.009[a]
Negative	60	
Positive	17	
LN metastasis		0.28
Negative	48	
Positive	29	
Distant metastasis		0.12
Negative	19	
Positive	58	
Dead status		0.04[a]
Alive	25	
Dead	52	
Recurrent status		0.09
No recurrence	29	
Recurrence	48	

[a]Statistical significance (*P*-value less than 0.05).

IPG 2D PAGE

IPG dry strips were equilibrated for 12–16 h in a solution containing 7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropy) dimethylammonio]-1-propanesulfonate, 1% dithiothreitol (DTT) and 1% pharmalyte. The equilibrated strips were loaded with 200 μg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences) following the manufacturer's instructions. For IEF, the voltage was linearly increased from 150 to 3500 V over 3 h (to allow for sample entry), then the voltage was held constant at 3500 V with the focusing complete after 96 kVh. Prior to the second dimension, the strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol) with 1% DTT and 2.5% iodoacetamide. The equilibrated strips were then inserted onto SDS-PAGE gels (20–24 cm, 10–16%) that were run using a Hoefer DALT 2D system (Amersham Biosciences) following the manufacturer's instructions. The 2D gels were run at 20°C for 1700 Vh, then silver stained by the fixing and sensitization steps with glutaraldehyde being omitted.

Image Analysis

Quantitative analyses of the digitized images were performed using the PDQuest software (version 7.0; BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized to the total valid spot intensity. Protein spots were identified as having a significant variation in expression if their expression level deviated by more than twofold relative to the expression level detected in the control or normal sample.

MALDI-TOF MS

A total of 64 gel spots (nucleus: 31, cytoplasm: 33) were excised from preparative gels using biopsy punches and transferred to a 1.5 ml siliconized Eppendorf tube. The gel spots were destained in destaining solution containing 100 mM $Na_2S_2O_3$ (Sigma) and 30 mM $K_3Fe(CN)_6$ (Sigma) (V/V, 1:1). Destained gel pieces were pre-reduced with 100% acetonitrile (HPLC grade). Then the protein-containing gel spots were reduced in reduction buffer (100 mM NH_4HCO_3 (Sigma) and 10 mM DTT (Sigma)) for 30 min at 56°C and then alkylated in

alkylation buffer (100 mM NH_4HCO_3 (Sigma) and 55 mM iodoacetamide (Sigma)) in the dark for 25 min at room temperature. The gel pieces were dried using a Speed-Vac. The dried gel pieces were incubated in ABC buffer (50 mM, pH 8.0) containing 0.1 μg/μl trypsin for 12–16 h at 37°C. Following concentration, the peptide mixture was desalted using C_{18}ZipTips (Millipore), and the peptides were eluted in 1–5 μl of 50% ACN/0.1% TFA. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% ACN/0.1% TFA, and 1 μl of this mixture was spotted onto a target plate. The tryptic peptide mixture was extracted and purified using a Millipore ZipTip C18 column and concentrated using ZipTips (Millipore Corp.). The peptide samples were cocrystallized with matrix on the Voyager 96 × 2 sample plate (P/N V700813) using 0.5 μl matrix (α-cyano-4-hydroxytranscinnamic acid) and 0.5 μl sample. The samples were then analyzed using the Applied Biosystems Voyager System 4307 MALDI-TOF Mass Spectrometer (ABI), and des-Arg[1]-Bradykinin, Glu[1]-Fibrinopeptide B and ACTH (clip 18–39) served as external standards for the mass calibration.

Clinical Survey for Tested Patients

We examined the medical records and archival slides in the collection of ovarian serous adenocarcinoma in the Gynecologic Oncology Files of Yonsei University College of Medicine in Korea. We selected 73 cases of ovarian serous carcinomas since 1990 for further review. Demographics (including age, sex, tumor site, tumor size, number of tumors, frequency of tumor recurrence and progression, associated malignancy and survival time) were obtained from each patient's medical records. The time to recurrence was calculated from the time of complete response (ie, no evidence of disease) to the time of the first tumor recurrence upon follow-up examination. All patients underwent total abdominal hysterectomy with both salpingo-oophorectomy and pelvic lymphadenectomy, including omentectomy. Patients with advanced ovarian cancer (stage III/IV) were also given postoperative chemotherapy.

The medical records of all tested patients were surveyed for the following information: age, stage of carcinoma, grade, ascites with positive peritoneal cytology, preoperative serum CA 125, postoperative tumor residue (2 cm), bilaterality, peritoneal implants, lymph node involvement and distant metastasis. The staging was in accordance with the standards of the International Federation of Gynecology and Obstetrics. Optimal cytoreduction was defined as less than 2 cm of postoperative tumor residue. Disease-specific survival was calculated using the Kaplan–Meier method, and statistical analyses were performed using the log-rank test. The Cox proportional hazards model was used for multivariate parameter analyses, and a stepwise model was used to select independent prognostic factors. A *P*-value of less than 0.05 was considered to be statistically significant. All statistical data were analyzed using SPSS (SPSS ver. 12.0; Chicago, IL, USA).

Tissue Specimens and Preparation of Samples for Immunohistochemistry

Seventy-three samples of ovarian serous carcinomas isolated from 1990 to 2003 were available for immunohistochemical analysis and censored data. Recipient blocks were made from purified agar in 3.8 × 2.2 × 0.5 cm frames. Using a core needle, 2-mm holes were made in these recipient blocks, and the resulting agar cores were discarded. The paraffin donor blocks were prepared following a thorough evaluation of the hematoxylin–eosin-stained slides. Two adjacent areas of a carcinoma from the matching donor blocks were transplanted to the recipient blocks using a 2-mm core needle. We constructed an array of samples of adjacent normal areas from these patients using paraffin-embedded, formalin-fixed tissue blocks. The recipient blocks were framed in the same mold that was used for the conventional paraffin block. Paraffin was then added to the frame. Consecutive 4-μm thick sections were cut from the recipient blocks using an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ, USA).

The 4-μm sections were placed on silane-coated slides, deparaffinized, immersed in phosphate-buffered saline (PBS) containing 0.3% (v/v) hydrogen peroxide and processed in a microwave oven (in 10 mM sodium citrate buffer, pH 6.5, for 15 min at 700 W). After blocking with 1% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween 20 for 30 min, the slides were incubated overnight at 4°C with nm23. Immunoperoxidase staining was performed using the streptavidin–biotin peroxidase complex method (LSAB universal kit; DAKO, Carpinteria, CA, USA). As negative controls, subtype-matched normal mouse IgGs were used. The final reaction product was visualized with the addition of 0.03% (w/v) of 3,3′-diaminobenzidine tetrachloride for 5–20 min. Strong cytoplasmic staining was considered a positive result. Immunostaining was graded and scored as follows: 0 (no staining), 1+ (weak, diffuse staining) and 2+ (strong, diffuse staining).

Database Analysis

The MS-Fit (UCSF Mass Spectrometry, ProteinProspector v 4.0.5) was used for protein identification via peptide mass fingerprinting. The spectra were calibrated using trypsin autodigestion ion peaks *m*/*z* (842.510, 2211.1046) as internal standards. Swiss-Prot. 10.30.2003 and NCBInr. 10.21.2003 were used as databases.

Results

Proteome Analysis of Human Ovarian Tumor Tissues

The 2D-E protein patterns were quantified and matched. To select protein variations, the protein patterns of the tumor and control samples were divided into two classes, and the quantities of all of the detected spots in each class were compared using the PDQuest software (v 7.0). To compare the differences in protein expression specifically in ovarian tumors, we included data from other tumors, including lung cancer, hepatoma, cervical cancer and renal cell carcinoma. The PMF maps were then obtained using MALDI-TOF MS. These data were used to search MS-Fit (ProteinProspector v 4.0.5), and the identity of protein was determined by comprehensively considering the corresponding experimental MOWSE score, the number of matched peptides Mp PI and the sequence coverage. Comparisons of spot expressions in the tissues analyzed revealed that all of the tumors tested (kidney, lung, uterine cervix and ovary), with the exception of hepatoma, had significantly higher *nm23* expression than their corresponding normal tissues. The *nm23* was more highly expressed in serous and endometrioid types of ovarian cancer and was more weakly expressed in mucinous types of ovarian cancer (Figure 1).

Clinical Correlation of nm23 Immunoreactivity

Ovarian serous carcinomas had positive immunoreactivity for *nm23* (Figure 2). The clinical correlation with *nm23* expression is illustrated in Table 1. The strong expression of *nm23* was inversely related to the presence of peritoneal implants. Specifically, 60% of the patients with strong *nm23* expression had no peritoneal implants, whereas only 27% of those patients had peritoneal implants ($P=0.009$). In addition, a stronger level of *nm23* expression was detected in patients with higher stages (III/IV) of disease than in those with lower stages (I/II) of the disease, but these differences are not significant ($P=0.06$). *Nm23* overexpression was significantly more frequent in dead patients of ovarian cancer than in alive patients (54 *vs* 28%; $P=0.02$).

Survival Analysis and *nm23* Expression in Ovarian Cancer

Multiple parameters have been used to predict the biological behavior of ovarian serous cancer by univariate (Table 2) and multivariate survival analyses (Table 3). In multivariate analysis, poor prognostic factors of disease-free survival included tumor residue more than 2 cm ($P=0.02$), bilaterality ($P=0.01$) and peritoneal seeding ($P<0.01$), whereas poor prognostic factor affecting overall survival included peritoneal seeding ($P=0.05$). In Kaplan–Meier analysis, strong *nm23* immunoreactivity correlates with poor overall survival ($P=0.04$) but not with poor disease-free survival (Figure 3).

Discussion

Using proteomics and immunohistochemistry, we discovered that *nm23* overexpression correlates with distant metastasis and reduced disease-free and overall survival in ovarian serous carcinoma, whereas reduced *nm23* expression is strongly

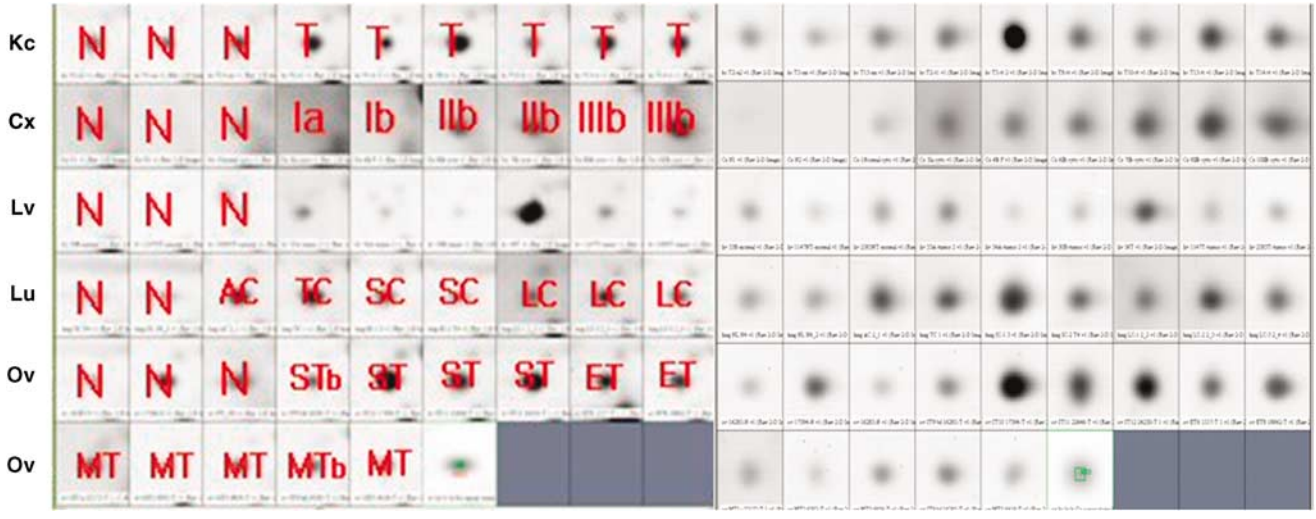

Figure 1 Comparative quantification in 2D-E protein spot. This spot was confirmed to be *nm23A* expressed in isoform A using MS-Fit. The above figure represents legend of each row and column corresponding to the below figure. Notice the strongest spot in ovarian serous carcinoma relative to endometrioid and mucinous tumors. There is an increase in *nm23* expression in cervical cancer, lung cancer and renal cell carcinoma, whereas there is a decrease in *nm23* expression in hepatoma. Cx, cervical cancer; Kc, kidney cancer; Lu, lung cancer; Lv, liver cancer; N, normal; Ov, ovary cancer; T, tumor. Each roman number in the Cx (cervical cancer) represents FIGO stage. ET,endometrioid carcinoma; MT, mucinous carcinoma; ST, serous carcinoma; STb, serous tumor of borderline malignancy.

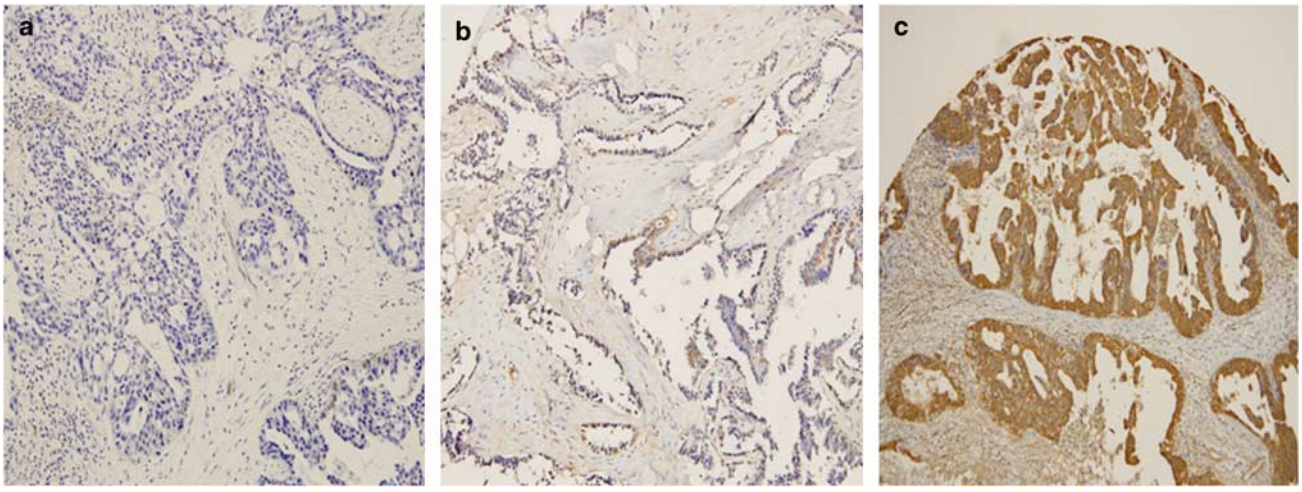

Figure 2 Immunohistochemical results of *nm23* in ovarian serous adenocarcinomas. (**a**) Negative immunostaining for *nm23*. (**b**) Focal, weak staining for nm23, graded as 1+. (**c**) Intense, strong immunoreactivity for *nm23*, graded as 2+ (which is regarded as a true positive result).

Table 2 Univariate survival analysis of nm23 overexpression in ovarian serous carcinoma

Variables	*Median survival (years)*	*HR (95% CI)*	P-*value*
Nm23		1.30 (1.01–1.68)	0.04*
Negative	Not reached		
Positive	4.50		
	Disease-free survival (years)	*HR (95% CI)*	P-*value*
Nm23		1.17 (0.96–1.42)	0.11
Negative	2.00		
Positive	1.25		

*Statistical significance (*P*-value less than 0.05).

Table 3 Multivariate survival analysis of nm23 overexpression in ovarian serous carcinoma

Variables	*HR (95% CI)*	P-*value*
Overall survival		
Peritoneal seeding	47.60 (1.04–2421.7)	0.05[a]
Disease-free survival		
Residual volume: non-optimal[b]	2.09 (1.13–3.87)	0.02[a]
Ovarian bilateralness	3.36 (1.31–8.62)	0.01[a]
Peritoneal seeding	3.87 (1.74–8.62)	<0.01[a]

[a]Statistical significance.

[b]Non-optimal: residual volume ≥2 cm.

associated with peritoneal implants. On the basis of these results, we propose that the differential expression of *nm23* affects its biological role. The association of *nm23* overexpression with distant metastasis is surprising, given the traditional view of *nm23* as a metastasis suppressor gene. Although this finding is contradictory, it is meaningful because it represents the first analysis of the role of *nm23* in ovarian cancer tissue samples using a proteomics approach involving well-designed ovarian serous cancer patients with a long-term follow-up period up to 13 years. These proteomics results, which were confirmed by immunohistochemistry, support our hypothesis that *nm23* overexpression is a tumor progression marker. Furthermore, we found a greater overexpression of *nm23* in serous carcinomas than in mucinous tumors. This result contradicts previous reports of *nm23* immunoexpression in ovarian mucinous tumors.[5] This discrepancy is likely due to the different interpretations and methods used in the studies. However, it should be noted that the immunohistochemistry of *nm23* should be interpreted cautiously as positive staining is restricted only to strong, diffuse expression not to focal, diffuse weak staining.[6]

The murine *nm23* cDNA was first identified by differential colony hybridization, which demonstrated its elevated expression in nonmetastatic murine K-1735 melanoma cell lines (nonmetastatic clone 23) and its reduced expression in a highly metastatic paired cell line.[7] In humans, there are two nm23 genes, *nm23-H1* and *nm23-H2,* that are located on chromosome band 17q–21.3. These genes encode human nucleoside diphosphate kinase A and B, respectively,[8] and share significant homology with the *Drosophila* development gene *awd* and the *Dictostelium NDP kinase* gene.[9] Expression levels of *nm23* have been widely reported in human tumor cohorts.[10] Although the expression of *nm23* is not a universally independent predictive or prognostic factor for ovarian cancer, both metastasis suppression and disease progression have been linked to elevated *nm23-H1* gene expression in different types of human tumors. As the simple overexpression of *nm23* is a poor prognostic factor, it is possible that *nm23* plays some tissue-specific roles and that different mechanisms regulating its overexpression

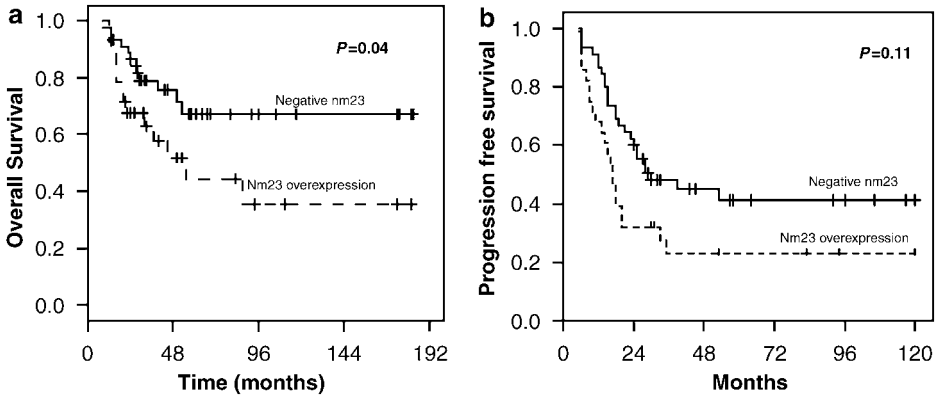

Figure 3 Kaplan–Meier survival with respect to *nm23* expression. (**a**) On overall survival analysis, *nm23* overexpression was significantly associated with poor survival ($P = 0.04$). (**b**) The overexpression of *nm23* was significantly associated with poor progression-free survival ($P = 0.03$). The continuous line represents the low expression of *nm23*, and the dotted line represents the high expression of *nm23*.

are involved in the development of certain types of tumors. Many studies have suggested an association between low *nm23* expression and tumor aggressiveness and patient mortality in hepatoma,[10] head and neck cancer,[11,12] melanoma[13] and breast cancer.[14,15] Despite the widely accepted data on reduced *nm23* expression and metastatic aggressiveness in breast cancer, it still does not represent an independent prognostic factor.[6] Furthermore, there has been no correlation or positive association between *nm23* expression and tumor aggressiveness reported in neuroblastoma,[16] genitourinary tract cancers (including testis, prostate and endometrium),[6] kidney cancer, thyroid cancer,[17] pancreatic cancer[18] or lung cancer.[19] The role that the *nm23-H1* gene plays in ovarian carcinoma has not yet been determined. The correlation between *nm23* expression and ovarian cancer was first examined in a cohort study in 1994.[20] This study demonstrated that the overexpression of both *nm23-H1* and *nm23-H2* was associated with the progression of malignant tumors rather than with the progression of normal ovary or benign tumors. Subsequent studies, using other methods such as northern blotting or immunohistochemistry, confirmed *nm23* as a tumor progression marker.[21–24] One report used immunohistochemistry to reveal a connection between *nm23-H1* overexpression and both survival advantage and greater response to chemotherapy in 106 patients with advanced ovarian cancer. In this study, there were more *nm23-H1*-positive patients without lymph node involvement (70%) than *nm23-H1*-positive patients with lymph node involvement (40%).[25] Later studies using allelic deletions of 17q revealed that an extremely high frequency of LOH (73–93%) had no impact on the prognosis or metastatic behavior of ovarian cancer.[21,22]

Despite extensive research, the biological mechanisms by which elevated *nm23* expression suppresses metastasis are still not understood. The interaction between *nm23-H1* and *Ksr* has been characterized,[26] and it was shown that mutations that inactivate *Ksr* suppress the phenotypic effects of activated Ras.[27] It is known that the overexpression of *nm23-H1* results in diminished Map kinase activation, which is required for higher histidine protein kinase activity.[28] Several characterized intracellular functions of *nm23*, namely microtubule assembly and disassembly,[29] signal transduction,[30] transcription regulation[31] and cellular adhesion,[32] are known to influence certain metastatic processes.

In other biological contexts, increased *nm23* expression correlates with aggressive disease. In these cases, the increased expression is associated with *nm23* mutations,[16,33] heterogeneity of phosphorylated isoforms,[34] association with GTPase-activating proteins[35] and NDP kinase activity.[36] The *nm23* mutations have seldom been detected in ovarian cancer. In a stratified univariate analysis and a complete multivariate model of 247 ovarian cancer patients, it was found that *nm23* overexpression was an independent predictor of an ominous outcome.[37] Schneider *et al*[9] demonstrated that *nm23* overexpression had a negative impact on prognosis and that the *nm23* overexpression was to a wild-type *nm23* gene rather than to mutant variants. It is possible that *nm23-H1* functions similarly to *nm23-H2*, which has been shown *in vitro* to initiate transcription of c-myc,[38] or another related oncogene associated with more malignant phenotypes in human ovarian cancer.

The present study to document that overexpression of *nm23* was significantly fewer in case of peritoneal seeding (27 *vs* 60%) represents that *nm23* expression suppresses peritoneal seeding. Regardless of peritoneal seeding suppression, however, *nm23* expression has shown that it was significantly associated with poor overall survival.

In conclusion, elevated *nm23* protein expression is an independent prognostic marker for diminished overall survival in patients with ovarian serous carcinoma.

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