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SPECIAL ARTICLE

Correlation of NM23-H1 cytoplasmic expression with metastatic stage in human prostate cancer tissue

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Abstract Nm23-H1 has been identified as a metastatic suppressor gene in murine melanoma cell lines. Several functions have been attributed to its activity in cancer, including a histidine kinase activity, DNA repair, and regulation of other proteins involved in metastatic formation. While in breast cancer, NM23-H1 overexpression indicates a benign status through impairing progression of disease, its function is opposite in other cancers; e.g., neuroblastoma. To further understand this dichotomy of function in cancer, we have analyzed its function in prostate cancer, in which the relationship between NM23-H1 expression and prognostic state is today controversial. In vitro, overexpression of NM23-H1 in PC3 cells inhibited their cell motility, while downregulation of NM23-H1 expression in these cells by RNA interference showed enhanced cell motility. Immunohistochemistry analysis performed on 346 prostate cancer tissue samples showed a relationship between high levels of NM23-H1 expression in the nuclei of these tumorigenic cells and elevated Gleason score, with high levels of NM23-H1 cytoplasmic staining related to metastatic stage. This retrospective survival study demonstrates that high levels of NM23-H1 expression in the cytoplasm determine recurrence of prostate-specific antigen levels only in those patients with metastatic disease. Our findings suggest a correlation between high levels of NM23-H1 protein in the cytoplasm of the cells and progression of prostate cancer to metastasis, thus definitively identifying NM23-H1 as a new negative prognostic marker in prostate cancer.

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Introduction

Prostate cancer is the most frequent cancer in the world, and the second greatest cause of death in American men. Despite the increasingly widespread use of prostate-specific antigen (PSA) screening, approximately 85% of cases of prostate cancer are diagnosed after the age of 65 years (Patel and Klein 2009; Pienta and Loberg 2005).

Huggins and Hodges were the first to define the use of androgen deprivation as firstline therapy for metastatic prostate cancer, with up to 80% of patients with metastatic disease responding to some form of androgen withdrawal (Huggins and Hodges 2002). However, the majority of men with metastatic prostate cancer ultimately progress to an androgen-independent state, which can result in death due to widespread metastases. Thus, metastatic prostate cancer proceeds through a series of distinct states, including transformation of normal prostatic epithelial cells to preinvasive primary tumor cells, androgen-dependent invasive cancer, and androgen-independent metastatic disease.

Responsiveness of the normal and malignant prostate to androgenic stimulation is determined by the presence of the androgen receptor in the cells; malignant prostate tumors respond to androgen ablation by retardation of growth. Removal of androgen can be achieved either by castration or by administration of luteinizing hormone-releasing hormone analogs; these agents lead to inhibition of testosterone production. As well as androgen ablation, endocrine approaches can be aimed at the control of growth of prostate cancer using nonsteroidal androgen-receptor antagonists, like hydroxyflutamide and bicalutamide (Culig et al. 2005).

The stages of prostate cancer involve multiple molecular changes that can be implicated in alterations in the expression of many genes. The identification of the genes that are directly regulated is an important approach to identify new prognostic markers. Although several genes have been identified and tested as diagnostic and prognostic markers for prostate cancer, to date, only PSA is routinely used in diagnosis (Chakrabarti et al. 2002). However, the use of PSA as a cancer-specific diagnostic test has well-recognized limitations. Many unnecessary biopsies are performed because in some cases high PSA values reflect a large prostate volume rather than a high risk of prostate cancer. Moreover, the use of PSA rate, PSA density and the free-to-total PSA ratio has provided only marginal improvements in terms of specificity. These limitations of PSA contribute to the ongoing debate about the benefits of population-based screening for prostate cancer (Ploussard and de la Taille 2010).

The first metastatic tumor-suppressor gene, NM23-H1, was identified by Steeg et al. (1988) through differential colony hybridization with seven cell lines derived from murine melanomas. This gene is located in chromosomal region 17q21.33 and the NM23-H1 protein has been proposed to have several biochemical functions, including its association with other proteins, which might, in turn, influence its antimetastatic function (Galasso and Zollo 2009). In humans, 10 genes have been identified as part of the Nm23 gene family (also known as the *NME* genes). Of these, the two most abundantly expressed are NM23-H1 and NM23-H2, which encode the A and B subunits of nucleoside diphosphate kinase (NDPK), respectively (Boissan et al. 2009; Lombardi et al. 2000). Several studies have shown correlations between reduced NM23-H1 expression and high tumor metastatic potential in several tumors types, such as liver, melanomas, colon, breast, ovarian, gastric, and hepatocellular carcinomas. In contrast, in other human carcinomas, such as thyroid, pancreatic and squamous cell lung carcinomas, and neuroblastoma and acute myelogenous leukemia, high mRNA and protein levels of NM23-H1 have been detected in aggressive tumors (Boissan et al. 2010; Lim et al. 1998; Okabe-Kado et al. 2009). In prostate cancer, the data present in the literature are contradictory. Igawa et al. showed that NM23-H1 protein expression correlates with increased proliferation status, and not to decreased metastases development, as investigated by immunohistochemistry analysis of human prostatic adenocarcinoma (Igawa et al. 1994). They concluded that the levels of NM23-H1 mRNA were specifically increased under proliferative conditions in prostate cell lines, and the NM23-H1 protein is considered to have pleiotropic effects on cellular proliferation and function in prostate cancer. In contrast, Konishi et al. (1993) showed that NM23-H1 expression (again detected by immunohistochemistry) was absent in metastatic lesions. In this study, where samples of 80 primary tumor tissues were analyzed, they showed positive immunostaining for NM23-H1 that indicated an inverse relationship between NM23-H1 expression and metastatic status. These data suggested that the nm23/NDP kinase might have a role in suppressing malignant potential in prostate carcinomas. In agreement with these data, Lee and Lee (1999) demonstrated that the NM23-H1 gene product suppresses the metastatic potential of prostate carcinoma cells by inhibiting their ability for anchorage-independent growth and for adhesion to the extracellular matrix in vitro.

Here, we have analyzed the opposing functions of NM23-H1 in cell motility and in metastasis progression in samples of prostate cancers from patients, through which we show a correlation between in vitro and in vivo data. Despite these inverse correlations reported between in vitro and in vivo studies, we show that using immunohistochemistry, NM23-H1 expression is significantly higher in

patients who later develop metastatic prostate cancer. Furthermore, we have analyzed the relationships between NM23-H1 expression and recurrence of increased PSA levels to determine whether the expression levels of NM23-H1 can provide a useful prognostic marker for patients with prostate cancer. These results shed light on the use of NM23-H1 as a new prognostic marker in combination with recurrence of increased PSA levels for the prediction of outcome for patients with prostate cancer.

Materials and methods

Cell culture

Androgen-independent PC3 and DU145 cells (Kaighn et al. 1979), androgen-sensitive LNCap cells (Horoszewicz et al. 1983), three human prostate cancer cell lines were grown in DMEM supplemented with 10% fetal calf serum (FCS). All media contained 100 units/ml penicillin, and 0.1 mg/ml streptomycin. PC3, LNcap and DU145 cells were cultured at 37°C in an atmosphere of 5% CO_2.

Real-time PCR analysis

Wild-type PC3, DU145, and LNCaP cells were plated at concentrations of $7–9\times10^6$ cells in 75-ml flasks. They were detached using trypsin solution, and following several washes in 0.1× phosphate-buffered saline, their RNA was extracted and purified using TRIZOL® (Invitrogen, see manufacturer instructions). Relative quantitation of NM23-H1 mRNA was performed using the SYBR® Green PCR Master Mix (Applied Biosystem), following the manufacturer instructions.

The following primers were designed using the Primer Express program (Applied Biosystem):

NM23H1, forward: 5′-GAGACCAACCCTGCA GACTC-3′.
NM23H1, reverse: 5′-CAAGCCGATCTCCTTCT CTG-3′.

The primer pairs were on different exons to minimize the possibility of genomic DNA amplification. All of the reactions were normalized to glyceraldehyde-3-phosphate dehydrogenase and quantitation was performed using the comparative CT method (Pfaffl 2001). A nontemplate control was run with every assay, and all of the determinations were performed in at least duplicates, for reproducibility.

Western blotting

Nuclear and cytoplasmic extracts were prepared from the PC3 and DU145 cells. The cells were washed several times with phosphate-buffered saline and harvested by centrifugation. The pellets were resuspended in 150 μl hypotonic buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1× protease inhibitor cocktail, 10% glycerol). After a 5-min incubation on ice, the cell suspensions were centrifuged at 900×*g* for 5 min. The supernatant (cytoplasmic) protein concentrations were determined using the Bio-Rad protein assay. The nuclear pellet was resuspended and incubated for 1 h in hypertonic solution (10 mM HEPES, pH 7.9, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM KCl, 1× protease inhibitor cocktail, 10% glycerol). After a 30-min centrifugation (14,000×*g*), the supernatant protein concentration was determined using the Bio-Rad protein assay. The samples were boiled for 5 min and 40 μg protein was analyzed by SDS-PAGE on 10% (*w*/*v*) or 12.5% (*w*/*v*) polyacrilamide gels. The proteins were electro-blotted onto PVDF membranes (Immobilon-P, Millipore). NM23-H1 was immunodetected with an anti-NM23-H1 monoclonal antibody (clone NM301; Santa Cruz) diluted 1/1,000 and the β-actin and β-laminin monoclonal antibodies monoclonal antibody (Upstate Biotechnology) diluted 1/500. After incubation with horseradish peroxidase-labeled anti-mouse IgG, visualization was by enhanced chemiluminescence (Amersham). The quantitation of the proteins in the different cellular compartments was by chemiluminescent detection using ChemiDoc XRS (Chem-iDoc™ XRS System, PC from Bio-Rad), performed with the Quantity One ® Software.

Transfection of PC3 cell lines

Full-length NM23-H1 cDNA was cloned into the mammalian expression vector pCDNA3.1 (Invitrogen). A small interfering sequence (siRNA) for NM23-H1 (si-nm23H1) was cloned into the pRetroSuper mammalian expression vector. The nucleotide sequences of this si-nm23H1 were as follows:

si-nm23H1, sense: ACCTGAGGAACTGGTAGATTAC
si-nm23H1, antisense: GTAATCTACCAGTTCCT CAGGG

The PC3 cells were transfected with these vectors using the TransFectin Lipid Reagent (BioRad) as described by the manufacturer.

In vitro cell motility assay

The PC3 cells were transiently transfected with the pCDNA3.1 CTR, pCDNA3.1 NM23-H1, pRetroSuper CTR, and pRetroSuper si-nm23H1 vectors and analyzed 48 h after transfection in the cell motility assays using a Boyden chamber (D'Angelo et al. 2004). Cell migration assays were performed with 8-μm pore size Transwells (Costar), with 5×10^4 cells suspended by trypsinization,

washed, resuspended in 100 μl serum-free DMEM containing 0.1% BSA, and placed in the upper chamber of the Transwells. The lower chamber was filled with 600 μl serum-free DMEM supplemented with 1% FCS, as the chemoattractor. The cells were allowed to migrate for 5 h at 37°C. Cells migrating to the lower side of the polycarbonate membrane were fixed with 2.5% glutaraldehyde and stained with hematoxylin solution (Sigma). These cells were counted under optical microscopy. Analysis was performed on three independent Transwells for each condition and each experiment was repeated twice.

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. All patients provided written consent for the collection of samples and the subsequent analysis. All of the human samples were de-identified through use of a numerical code.

Immunohistochemistry analysis

We also carried out immunohistochemistry analyses on prostate cancer tissue microarrays (TMAs) with all the follow-up data by Drs. Andre Balla and Milton Datta for the Cooperative Prostate Cancer Tissue Resource. This included a total of 300 prostate cancer cohorts and additionally another 46 patients, representing all of the disease stages, which were related in this study (including Gleason score) of prostate cancer progression collected by Dr. Staibano ("Federico II" University of Naples). These immunohistopathology analyses included cellular compartment evaluation (nuclear and cytosol staining) and were performed by three independent pathologists (Dr. Arrigoni, Dr. Staibano, and Prof. Troncone). The clinico-pathological characteristics of the patients analyzed in the TMAs are summarized in Supplemental Table S1.

The primary anti-NM23-H1 antibody, clone 37.6 (Novocastra), was incubated for 2 h at room temperature at a dilution 1:300. Subsequently, the signals were detected using the NovoLinkTM Polymer Detection System (Novocastra Laboratories Ltd, distributed by A. Menarini Diagnostics). Tumors were considered positive if at least 10% of the tumor cells were stained. Signal intensity was grouped as follows: negative (0, expression in <10% of cells), weakly positive (1, +), moderately positive (2, ++), and strongly positive (3, +++); in addition, the percentage of nuclear (N) and/or cytoplasmic (C) tumor cell immunoreactivity was also monitored.

Statistical analysis

The two-sided Fisher's exact test was used to verify the associations among the protein levels of NM23-H1 and the clinicopathological variables. The effects of NM23-H1 overexpression on survival were analyzed according to the Kaplan–Meier method and the significance of the differences in survival was determined using the log-rank test.

We divided the patients into two groups on the basis of NM23-H1 protein expression: −/+ NM23-H1 indicates patients with low NM23-H1 protein expression; and ++/+++ NM23-H1 indicates patients with overexpression of the NM23-H1 protein. $P<0.05$ was considered as statistically significant.

The following variables and categories were defined and included in our analyses: age at diagnosis, pathological primary tumor size, Gleason score (≤6, 7, ≥8), pathological nodal status, presence of metastases, and overall survival (from time of diagnosis). Some of these variables were missing for some of the patients. In particular, nodal status, presence of metastases, and receptor status were not known in a large fraction of the cases, as these variables were not required for the inclusion of the patients in the study. The exact coefficient for sample proportion analysis was calculated to determine all of the significant variables (<0.05 level).

Results

NM23-H1 expression in prostate cancer cell lines

The expression levels of NM23-H1 were evaluated by real-time PCR in three different prostate cancer cell types (PC3, LNcap, and DU145 cell lines; Fig. 1a and Supplemental Table S2 for CT values). Here, the PC3 cells had increased levels of NM23-H1, although the differences across these three cell lines were not significant. To further study the expression of NM23-H1, we analyzed the nuclear and cytoplasmic expression of NM23-H1 by Western blotting in PC3 and DU145 prostate cancer cell lines (Fig. 1b, c). The NM23-H1 protein was expressed in both the cytosol and the nuclear compartment of these cells. Indeed, these cell lines represent two models for high metastatic phenotype in prostate cancer (see Table 1). The PC3 cells have a higher metastatic potential with respect to the DU145 cells, and these showed lower expression of NM23-H1. The nuclear expression of NM23-H1 indicated a potential function of this protein in the nuclear compartment, although as we have seen very recently (Steeg et al. 2011, special issue on NDPK, personal communication of Prof. M. Zollo), this nuclear function of NM23-H1 will be difficult to address. There remains the need for the design of suitable assays because of the contradictory and limited data that are available in the literature at this time. Following consideration of this situation, we decided at this stage that the investigation of this nuclear function at the molecular level

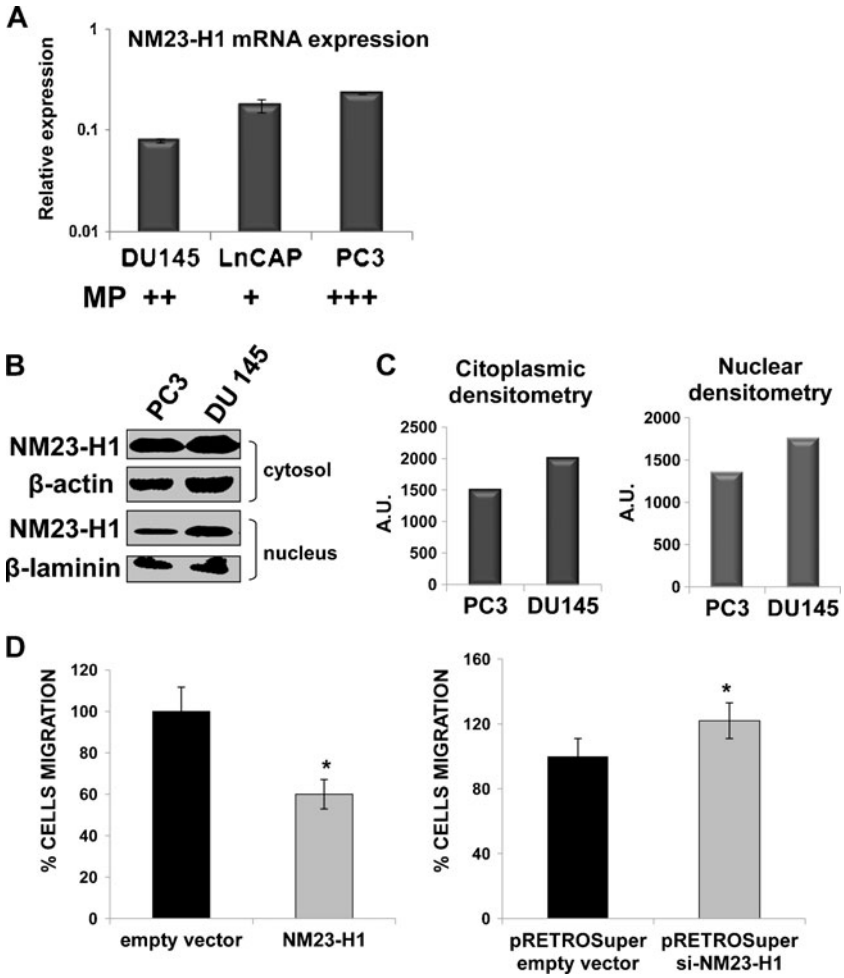

Fig. 1 NM23-H1 expression in prostate cell lines. **a** Real-time PCR analysis of nm23-H1 mRNA expression in different types of prostate cell lines. Expression levels of nm23-H1 are representative for any cell prostate line. Below each cell line is indicated the metastatic potential (MP) as shown in Table 1. All data are normalized against GAPDH. **b** Evaluation of nuclear and cytoplasmatic expression of nm23-H1 by Western blotting analysis in the prostate cell lines. **c** Densitometric analysis of nm23-H1 protein in the different cellular compartments performed using the Chemi Doc XRS System, PC from Bio-Rad. *A.U.* indicate arbitrary units. **d** Cell motility assay on PC3 cells overexpressing pCDNA3.1 nm23-H1 (*left panel*, **P*=0.006). Cell motility assay on PC3 cells silenced with pRetroSuper si-nm23-H1 (*right panel*, **P*=0.005)

is beyond the scope of the present study, and will thus be an issue for future studies.

Motility effects of NM23-H1 in PC3 cells

NM23-H1 expression is increased in breast carcinoma cell lines, and it has been demonstrated that this results in reduced cell motility and is correlated with aggressiveness of sarcoma and breast carcinoma tumors (Bemis and Schedin 2000; Palmieri et al. 2005). We studied the motility effects of NM23-H1 in the PC3 cell line, which represents a model for a high metastatic phenotype of prostate cancer. For this, we made use of the Boyden chamber assay, with 1% FCS as chemo-attractor, to investigate whether expression of the NM23-H1 gene results in inhibition or induction of metastatic function (measured here as cell migration) in the PC3 prostate cell line. As can be seen from Fig. 1d (left panel) this transient overexpression of NM23-H1 resulted

Table 1 Characteristics of the three types of prostate cancer cells (PC3, DU145 and LNCaP cells) used in the present study

	Growth properties	Morphology	Tumor type adenocarcinoma	Metastatic potential	Metastatic site	References
PC3	Adherent	Epithelial	+	+++	Bone	Kaighn et al. 1979
DU145	Adherent	Epithelial	+	++	Brain	Stone KR (1978) Int. J. Cancer 21: 274–281.
LNCaP	Adherent, single cells and loosely attached clusters	Epithelial	+	+	Left supraclavicular lymph node	Horoszewicz et al. 1983

(+) Low level, (++) moderate level, (+++) high level

in significant inhibition of cell migration (P=0.006), supporting a role for NM23-H1 as an inhibitor of cell motility in these PC3 cells. Conversely, when NM23-H1 was silenced using the siRNA si-nm23H1, there was significant enhanced cell motility in these PC3 cells (Fig. 1d, right panel; P=0.005). These data confirm that overexpression of NM23-H1 suppresses the migratory phenotype of this high metastatic cell line.

At this stage, we wanted to determine whether the expression of NM23-H1 in tumor samples correlates with prognosis of these positive prostate cancer-affected patients.

Immunohistochemistry analysis of NM23-H1 proteins in prostate cancer tissue multiple arrays

With cellular staining in both the nuclear and cytoplasmic compartments in these prostate cancer cells in vitro, we wanted to analyze these expression patterns in prostate tumor tissues. To this end, we carried out immunohistochemistry analyses with prostate cancer TMAs, including a total of 300 samples from patients with prostate cancers through the Cooperative Prostate Cancer Tissue Resource at the National Cancer Institute (NCI, Bethesda, USA) and other 46 tissue from classic sections. Here, we were able to assess the immunohistochemistry staining on multiple tissue arrays containing prostate cancer tissues that were accompanied by their full assessment according to stage of the disease (i.e., tumor node metastasis classification and Gleason score), along with 46 further prostate cancer tumor samples representing all stages of prostate cancer progression ("Federico II" University of Naples, Italy). Figure 2a shows representative immunohistochemistry staining for NM23-H1, with this data obtained from the tissue microarray series; Fig. 2b shows an example of the immunohistochemistry staining for NM23-H1 from one of the classic sections of prostate tumors. Overall, the staining showed positivity in both of the cellular compartments (nuclear and cytoplasm). Then, immunohistopathology analysis scoring was performed independently by three pathologists using a unified updated prostate cancer pathological staining correlation analysis, as described in the "Material and methods" section. These data were then analyzed for correlation with the pathological characteristics of these prostate cancer patients.

NM23-H1 protein expression and its correlation with the clinicopathology variables

We performed a survival analysis according to Kaplan–Meier methodology and expression of the NM23-H1 protein, which showed a significance correlation with survival, according to the log-rank test. These analyses thus showed correlation between high levels of the NM23-H1 protein in the cell cytoplasm under immunohistochemistry staining with a recurrence of increased PSA levels for these patients with metastases (Fig. 2c; P=0.0064).

Additionally, we performed statistical multivariate analysis of 346 of these patients, where we found nuclear NM23-H1 expression to be positively correlated with Gleason score ($p \leq 0.034$), and that high levels of the NM23-H1 protein in the cytoplasm were related to the metastatic stage (Fisher's exact test; P=0.0001; see Table 2). Statistical correlations to the nuclear staining of NM23-H1 were not addressed by any further analyses. Interestingly, in these patients of 65 years old (the mean age in this prostate cohorts dataset), we also found a significant relationship between high NM23-H1 staining intensity in the cell cytoplasm and recurrence of increased PSA after radical prostatectomy (P=0.018; see Table 3).

Discussion

Clinical and pathological risk factors, such as age, tumor size, and axillary nodal and steroid receptor status, are commonly used to assess the likelihood of metastasis development in patients with prostate cancer. Distant metastases are the main cause of death among many cancer patients (Weigelt et al. 2005).

NM23-H1 is predominantly expressed in the cytoplasmic compartment (Kraeft et al. 1996). We have shown here that the NM23-H1 protein is expressed in all of the prostate cell lines analyzed in this study. Additionally, nuclear compartment staining of prostate cancer cells and tissues was observed for the first time. At this stage, this nuclear function of NM23-H1 needs to be investigated further in patients with prostate cancer, as we can only speculate on its potential influence on the transcriptional machinery (Postel et al. 2000) or its exonuclease activity (Zhang et al. 2011). Kaetzel and colleagues (Ma et al. 2004) reported a 3′-5′ DNA exonuclease activity for NDPK-A/NM23-H1, and in a follow-up study, an E5A mutation of NDPK-A/NM23-H1 was reported to decrease its exonuclease activity without effects on its NDPK or histidine protein kinase activities. This mutation reduced the metastasis suppressor function of NDPK-A/NM23-H1 in vivo, although it did not show motility suppression in vitro (Zhang et al. 2011). Data in prostate cancer will be definitively needed to address these first observations here with this tissue immune staining.

Several studies have demonstrated a suppressive effect of NM23-H1 on the in vivo metastatic aggressiveness of melanoma and breast carcinoma cells (Leone et al. 1993; Parhar et al. 1995). In vitro analysis on NM23-H1-transfected melanoma and breast carcinoma cells revealed that NM23-H1 expression inhibits colonization, invasion, and motility of tumor cells (Kantor et al. 1993). Further studies have confirmed that restoration of NM23-H1

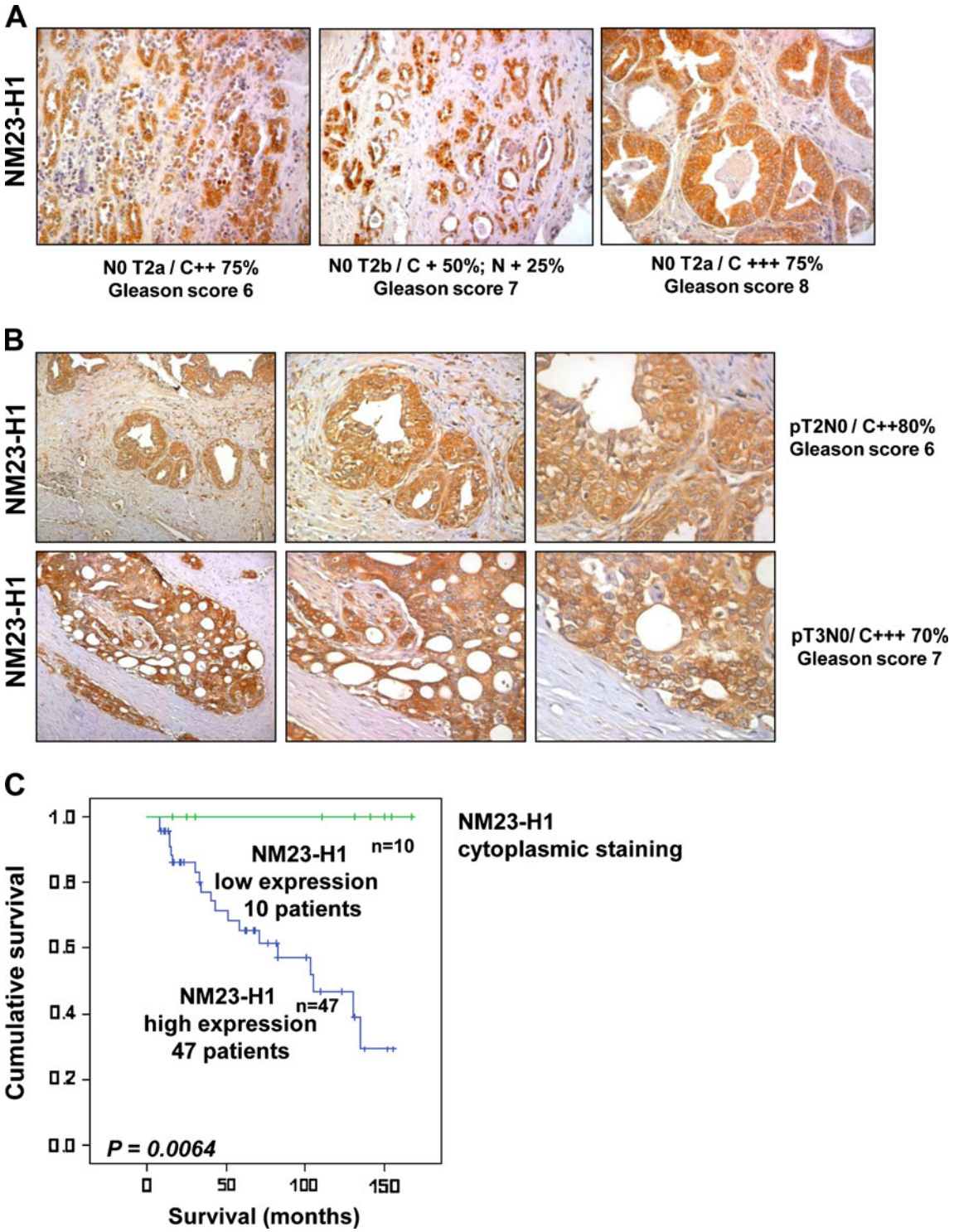

Fig. 2 Immunohistochemistry analysis of nm23-H1 in prostatic cancer tissues. **a** On the top panel, immunohistochemistry staining for NM23-H1 in some cases from a tissue microarray series (×25 magnification). **b** Immunohistochemistry staining for NM23-H1 in two cases from classic sections of prostatic tumor at three different magnification (×25, ×50, ×100). **c** Survival analysis according to Kaplan-Meier method. The patients are divided according to NM23-H1 low expression (*green line*) and NM23-H1 high expression (*blue line*) groups (P=0.0064)

expression reduces metastasis of breast, colon, prostate, and oral squamous cell carcinomas, and of melanoma tumor cell lines (Russell et al. 1997; Suzuki et al. 2004).

Our data indicate that NM23-H1 significantly inhibits the cell motility of PC3 cells, a cell model of metastatic prostate cancer, when these cells transiently overexpress the

Table 2 Association between protein expression levels of NM23-H1 (cytoplasmic and nuclear) and several pathologic variables in 300 patients: age of diagnosis, pathologic primary tumor size (pT1, pT2, pT3, and pT4), Gleason score, nodal status (N0, N+) and presence of metastases using Pearson's chi-square test

NM23-H1 staining	Overall (only evaluable cases)	Staining intensity cytoplasm			Overall (only evaluable cases)	Staining intensity nucleus		
		−/+ NM23-H1	++/+++ NM23-H1	*P* value		−/+ NM23-H1	++/+++ NM23-H1	*P* value
Variable								
Age								
≤65	132	49	83	1	132	107	25	0.2
≥66	140	52	88		140	104	36	
Pathologic stage								
pT1+pT2	165	57	108	0.3	165	129	36	0.77
pT3+pT4	107	44	63		107	82	25	
Gleason score								
≤6	85	33	52	0.88	85	73	12	0.034
7	156	56	100		156	118	38	
≥8	31	12	19		31	20	11	
pN								
N0	244	87	157	0.23	244	191	53	0.55
N1	8	3	5		8	5	3	
Nx	20	11	9		20	15	5	
pM								
M0	103	54	49	<0.0001	103	83	20	0.37
Mx	169	47	122		169	128	41	

NM-23H1 cDNA under a strong promoter. Conversely, after silencing NM23-H1 in these PC3 cells, their migration returned to their normal migratory phenotype, thus confirming NM23-H1 as a suppressor of motility functions in vitro in prostate cancer cells. In previous studies with other cancer types (i.e., melanoma and non-Hodgkin lymphoma), overexpression of NM23-H1 led to an increase in the metastatic potential at the cell level (Florenes et al. 1992; Hartsough and Steeg 2000; Niitsu et al. 2001). The results obtained in these prostate cancer cells are different to what we found in our ex vivo analysis in prostate tumor tissues, although at this stage we need to weight the ex vivo analysis in tumor tissues to be able to correctly judge its prognostic function. To clarify the role of NM23-H1 in prostate carcinoma, we analyzed here its protein expression in 346 patient tissues using immunohistochemistry. We found that NM23-H1 expression is correlated with an increased Gleason score when the protein is prevalently expressed in the nuclei of those tumorigenic cells: here, 48% of the patients analyzed with a Gleason score of 7 showed low levels of the NM23-H1 protein. One of the limitations of this analysis is that the Gleason scores were about 7 or above in the majority of our patients. For this reason, related to our study, the Gleason score pattern appears not to be sufficient to assess prognosis, with the histological pattern as the most important for the prediction of patient outcome. Despite this, overall, the Gleason grading provides an estimation of the biological aggressiveness of a prostate cancer. Indeed, the aggressiveness of prostate cancer is closely linked to the presence of a high-grade tumor

Table 3 Relationship between the high staining intensity of NM23-H1 in the nucleus and cytoplasm, and the PSA recurrence after radical prostatectomy in patients with age minor of 65 years

NM23-H1 staining	Overall (only evaluable cases)	−/+ NM23-H1	++/+++ NM23-H1	*P* value
Variable PSA recurrence	Staining intensity cytoplasm			
No recurrence	34	11	23	0.018
Recurred	53	14	39	
	Staining intensity nucleus			
No recurrence	34	31	3	0.21
Recurred	53	41	12	

(Gleason score=9; Cheng et al. 2005). According to the significance of our data, it appears that the increases in vitro of NM23-H1 expression in the nuclei of the prostate cancer cells is a sign of rising malignancy, as encountered in vivo by the Gleason score correlations in the tumor specimens and our above-reported statistical correlations. The function of NM23-H1 in the nucleus of these prostate cancer cells will be further analyzed in future studies.

At this stage, we can envision a potential epigenetic positive modification during tumorigenesis, such that the enhancing of NM23-H1 expression is coupled to some at present unknown mechanism of activation performed by "gene/protein controllers", which can then switch off its "suppressor of metastasis property" and induce an opposite "metastasis-promoting" function. These "controllers" might thus be protein binding partners that can act through their ability to change the status/ function of NM23-H1 during the progression of tumorigenesis. If this hypothesis is confirmed, we should find that this phenomenon occurs in other tumors where NM23-H1 has been found with an opposite function (as in the examples reported above). Future studies should thus address this concept and define the mechanisms of action that influence this dichotomy of function of NM23-H1.

We also show here that high levels of the NM23-H1 protein in the cell cytoplasm in the prostate cancer samples were related to the presence of distant metastasis. In prostate cancer, the presence or absence of distant metastases is a determining factor in the clinical outcome of this disease. In some patients, prostate cancer metastasizes rapidly and can kill the patient in less than a year, while other patients can live for many years with localized disease without signs of apparent metastases (Heidenberg et al. 1994). Since death from prostate cancer occurs after the development of metastases, as for many tumors, identification of markers that are involved in the metastatic process holds promise for the development of prognostic and therapeutic advances. We show a significant relationship between high levels of NM23-H1 protein expression in the cytoplasmic compartment and the recurrence of increases in PSA levels. PSA is one of the most used and accepted diagnostic and prognostic factors for patients with prostate cancer, with serum PSA levels greater than the range of 4–10 ng/ml strictly correlated to disease recurrence. Overall, these patients who show recurrence also have a poor prognosis. Moreover, using retrospective follow-up data and Kaplan–Meier survival analysis, we show here that high levels of the NM23-H1 protein into the cytoplasm are associated with greater recurrence of PSA increases only for the patients who were then reported to show metastases. Thus, the patients with high levels of NM23-H1 protein have a worse prognosis, indicating that the staining in prostate tumor samples for NM23-H1 before prostatectomy appears to be important in the diagnostic analysis for potential recurrence of PSA increases and worse patient survival.

Experiments in vitro mostly show the influence of the NM23-H1 protein in cytoskeleton remodeling, which can then have a negative influence on cell motility. While this phenomena seen in vitro would indicate a function for NM23-H1 as an inhibitor of tumor invasion, with the opposite seen in vivo, we can speculate that in tumorigenesis some other genes/ proteins are influencing this NM23-H1 function, thus linking its expression in primary tumors to a lower probability for recover from prostate cancer in these patients, even after resection of the prostate gland or hormone treatment. Here, we propose to use this marker before gland ablation or hormone treatment, to further categorize those patients who will need more invasive clinical treatment to inhibit the progression of their prostate cancer.

In future studies, we will also be addressing the possibility of determining whether these metastatic cells in patients with prostate cancer are delivered throughout the body of those affected, and whether they can be determined by analysis of the serum levels of NM23-H1 with NM23-H1-specific antibodies. These ongoing studies will enhance these findings and hold promise in particular for better treatment of these patients with metastatic prostate cancer.

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