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Clinical significance of *nm23* expression in renal cell carcinoma

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Abstract In many tumors an expression of *nm23* gene products is associated with a lower metastatic potential. The aim was to evaluate whether *nm23* gene expression in renal cell carcinoma was associated with clinicopathological findings and survival. In 41 patients, the expression of nm23 protein was analyzed in tumor and corresponding kidney cortex tissue by immunohistochemical analysis using a monoclonal nm23-H1 antibody. In all kidney cortex samples intense *nm23* staining was found. Of 41 tumors, 15 had high, 12 intermediate, 5 low nm23 expression whereas 9 tumors showed none. There were no differences in *nm23* staining between different stages, grades or size of tumor. No correlation between survival and *nm23* expression was observed. However, diploid tumors had significantly less *nm23* staining compared with aneuploid tumors, indicating that nm23 gene inactivation might be a favorable sign. The expression of *nm23* gene products seems not to be correlated to tumor progression and metastatic ability in renal cell carcinoma.

Key words *nm23* · Immunohistochemistry · Renal cell carcinoma · Flow cytometry · Survival

Introduction

Metastatic spread is the cause of death for most patients with malignant tumors. The progression and metastatic spread for patients with renal cell carcinoma has been difficult to predict due to a pronounced variety in the clinical behavior. About 30% of the patients have distant metastases at the time of primary diagnosis and patients without evidence of metastatic disease face a 40%–50% chance that metastatic disease exists and will become manifest within 5 years [25]. New molecular-based tests are therefore required to assist in the determination of the potential aggressive nature of different subgroups of renal cell carcinoma.

Considerable investigation has focused on genetic events participating in the formation of tumor metastasis. A putative metastasis suppressor gene, *nm23*, was identified in murine melanoma cell lines using differential screening between low and high metastatic cell clones [26]. In humans two genes *nm23-H1* and *nm23-H2* localized to chromosome 17q and corresponding to NDP kinase A and B have been distinguished [2]. A correlation of high nm23 protein and mRNA expression to a low metastatic potential has been established in several malignancies, based on published prognostic studies with tumor cohorts and transfection studies [16, 21, 22]. In primary human infiltrating ductal breast carcinomas, tumors from patients with metastasis to the lymph nodes had lower nm23 mRNA levels compared with non-metastatic [3]. In a variety of other solid tumors such as breast carcinoma [8, 27], melanoma [5], endometrial, cervical [21], ovarian [12], and gastric carcinoma [22], low *nm23* expression has been associated with metastatic phenotype.

Contradictory results, concerning the metastatic suppressive activity of *nm23* have been reported in a number of malignancies [6, 9, 18, 24], where increased *nm23* expression has been associated with disease progression. Moreover, amplification and mutation of the *nm23-H1* gene have shown to be correlated to advanced stage and poor patient survival [6, 17].

In renal cell carcinoma only a few studies on *nm23* have been performed and with divergent results [10, 29]. The aim of the present study was to evaluate further the association between *nm23* expression and clinicopathological findings, DNA ploidy and survival in patients with renal cell carcinoma.

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Materials and methods

Patients

The study comprised 41 patients with previously untreated renal cell carcinoma, operated upon with radical nephrectomy between January 1989 and November 1991. There were 24 men and 17 women, mean age 63.8 years, range 25–84 years. Histopathological nuclear grade was defined according to Skinner et al. [25] and stage was defined according to the 1992 TNM system. Preoperatively, the patients were examined with chest X-ray, ultrasound and computed tomography, and in patients with any symptom or laboratory sign of bone metastases with bone scans. Immediately after nephrectomy the tumor-bearing kidney was divided and tissue samples were taken from the tumor and corresponding normal kidney cortex. The samples were fixed in buffered formaline and paraffin embedded. At the end of follow-up, 9 patients were alive without evidence of the disease, 5 had died of intercurrent diseases while 27 patients had died of the disease.

Immunohistochemistry

The sections were deparaffinized in saline and then hydrated through graded alcohols. Briefly, after incubation for 15 minutes in 0.1% hydrogen peroxidase and washing in PBS, the slides were incubated with normal blocking serum in PBS to suppress non-specific binding of Igg. The slides were then incubated with a mouse monoclonal *nm23-H1* IgG_{2a} antibody overnight at 4°C (Santra Cruz Biotechnology, Santa Cruz, Calif.). The slides were washed three times with PBS and incubated for 30 minutes with a biotin-conjugated second-step antibody (Santa Cruz ABC ImmunoStain Systems) and thereafter with avidin biotin enzyme reagent. The sections were counterstained in haematoxylin-eosin. The rate and intensity of positive *nm23* staining was evaluated as the mean percentage of immunostaining in 20 different fields at a magnification of ×100 and graded: 0, no visible staining; +1, 0–30% stained cells; +2, 30%–60%; +3, 60%–100% stained cells. All evaluations were performed blind by one investigator.

Flow cytometric analysis

The method for flow cytometric DNA analysis has been outlined previously [13]. Briefly, immediately after the nephrectomy, one kidney cortex and six different tumor samples were taken. The fresh tissue samples were minced and stained using a propidium iodide solution and run in a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, Calif.). The tumor samples were denominated diploid when only one peak was detected and aneuploid when two separate peaks were found since it was assumed that all tumor samples contained normal as well as tumor cells. In each patient the kidney cortex tissue sample was used as an internal DNA ploidy standard. The proportion of S-phase cells was evaluated from DNA histograms using the R-fit program supplied from Becton-Dickinson.

Statistics

For statistical evaluation Fishers exact probability test and Mann-Whitney U-test were used. A P-value less than 0.05 was considered statistically significant. Survival time calculations were illustrated with Kaplan-Meier curves and analyzed with the log rank test to determine the level of significance.

Results

All normal kidney cortex tissues had a high frequent staining (3+) of the *nm23* gene product. Of 41 tumors, 15 stained 3+ (high, exemplified in Fig. 1a), 12 intermediate (2+), 5 low staining (1+), whereas 9 tumors had no *nm23* expression (0), (Fig. 1b). When subdividing the tumors according to tumor size (median diameter 8.0 cm), no difference in *nm23* expression between tumors larger and those smaller than median size was found. The relations between nm23 protein expression and tumor stage, grade and DNA ploidy are shown in Table 1. No correlation was found between *nm23* expression and tumor stage or nuclear grade.

Homogeneously diploid tumors had significantly less *nm23* staining compared with aneuploid tumors ($P = 0.004$). Six of 7 diploid tumors expressed low or negative *nm23* staining whereas 26 of 34 aneuploid tumors expressed more frequent staining. Tumors with low or negative *nm23* staining had significantly lower S-phase fractions than tumors with higher *nm23* staining ($P = 0.04$). No correlation between *nm23* expression and tumor progression was found. Neither was there any survival difference between patients with low or negative *nm23* staining compared with patients having tumors with intermediate or high *nm23* staining (Fig. 2).

Discussion

In renal cell carcinoma, tumor stage is the most significant prognostic factor, but for an individual patient the prediction of metastases remains difficult. In several tumors a correlation between high *nm23* gene expression and low metastatic potential has been established [3, 5, 8, 12, 21, 22, 27]. The potential role of *nm23* as a metastatic suppressor gene was supported by evidence for a functional involvement of *nm23* after transfection of nm23-H1 cDNA into murine melanoma cell line [15]. Also, transfection of highly metastatic human breast carcinoma cells with nm23-H1 cDNA resulted in a significant reduction in the metastatic potential in vivo [19]. Although the precise mechanism of action is unknown, several different explanations for the action of the *nm23* genes have been proposed. One possible mechanism for the antimetastatic action has been shown by in vitro inhibition of colonization and motility [11].

In contrast, the present study showed no correlation between the expression of nm23 protein and tumor progression and metastatic ability in renal cell carcinoma. The results are in line with findings obtained in prostate, bladder and colon carcinomas indicating no correlation of *nm23* expression to metastatic spread [6, 9, 18, 24]. Our data also support a previous investigation of renal cell carcinoma analyzing both nm23-H1 and nm23-H2 monoclonal antibodies. In that study no correlation to survival was found [18]. Furthermore Bosnar et al. [4], analyzing gene instability, found that *nm23-H1* does not play any key role in the invasiveness of renal cell carcinoma. Nm23 mRNA levels were equally expressed in human colon and renal cell carcinoma clones, regardless of their metastatic potential in nude mice [23]. On the other hand, using a polyclonal antibody

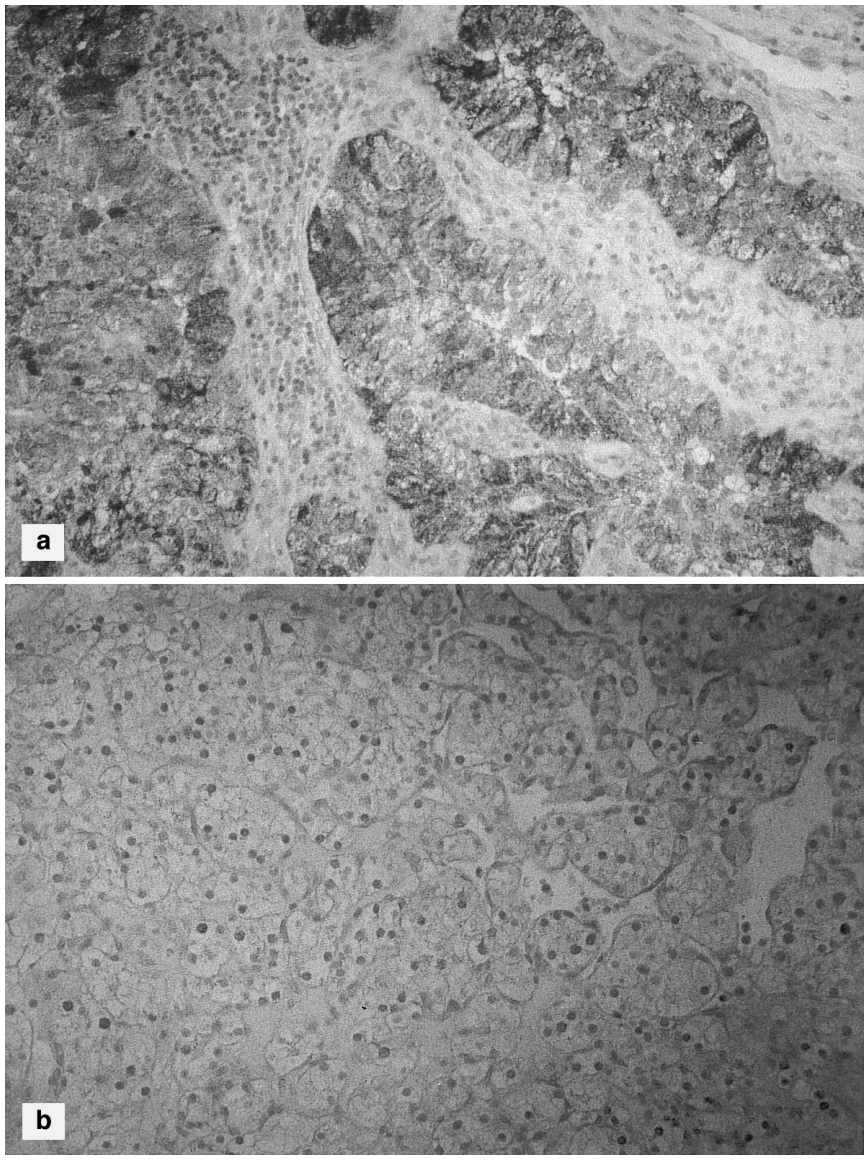

Fig. 1 Immunohistochemical staining of renal cell carcinoma with a monoclonal nm23-H1 IgG_{2a} antibody and binding was detected with an avidin-biotin system. **a** Represents renal cell carcinoma with 3+ staining and **b** no visible *nm23* staining

Urakami et al. [28] found a significant correlation of high nm23 protein expression with poor clinical outcome. Similar results were reported in a small study comparing grade 1 and 2 tumors, where a tendency towards higher *nm23* expression in grade 2 tumors was found [10].

Several papers have reported conflicting results from investigations of in vivo tumor material in other malignancies. In thyroid carcinoma the expression of nm23-H1 protein was low or absent in metastatic lymph nodes and bone marrow [1] whereas another study showed increased levels of *nm23* gene in advanced stages [30]. In ovarian cancer Leary et al. [14] showed a high rate of allele loss at the *nm23-H1* locus (73%) whereas Mandai et al. [20] found only loss of heterozygosity in 22% at the *nm23* locus.

The lower *nm23* expression in diploid renal cell carcinomas compared with higher *nm23* expression in aneuploid tumors confirms a previous study of colorectal carcinoma [18]. In that study, as well as in the present, no correlation between *nm23* expression and other tumor characteristics besides DNA ploidy and S-phase fractions was found. Diploid renal cell carcinomas generally have a lower proliferation rate and longer survival time compared with aneuploid tumors [13]. The low staining in diploid renal cell carcinoma might suggest that absent or low expression of *nm23* could be a favorable prognostic sign. Hiasa et al. [7] were, however, unable to find any correlation with proliferation when studying 95 renal cell carcinomas with immunohistochemistry. Our results confirm studies on prostate and bladder carcinomas showing higher *nm23* levels in tumors with a high proportion of cycling cells [9, 24]. However, the generally lower *nm23* expression in tumor tissues, especially in the diploid, compared with high frequent staining in normal kidney cortex tissues indicate that the *nm23* gene dysregulation might be an early genetic event in the evolution of renal cell carcinoma.

Table 1 *nm23* expression in relation to tumor stage, nuclear grade, DNA ploidy and S-phase fraction in 41 patients with renal cell carcinoma

	nm23 expression				Total
	0	+1	+2	+3	
Tumors (*n*)	9	5	12	15	41
Stage					
T1, T2, N0, M0	3	1	4	3	11
T3, N0-1, M0	3	–	3	5	11
T2-4, N0-1, M1	3	4	5	7	19
Grade					
1	1	–	–	–	1
2	1	–	1	3	5
3	4	4	5	8	21
4	3	1	6	4	14
DNA-ploidy					
Diploid	4	2	–	1	7
Aneuploid	5	3	12	14	34
S-phase fraction					
Mean ± SD	5.0 ± 3.2	6.7 ± 1.6	11.1 ± 5.9	7.6 ± 3.7	7.5 ± 4.5
Median	4.2	6.8	10.7	7.2	6.8

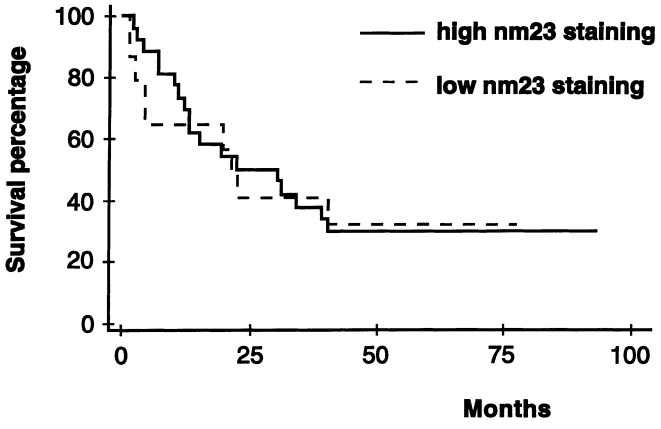

Fig. 2 Kaplan-Meier survival curves of 41 patients with renal cell carcinoma subdivided in 14 patients having low or no *nm23* expression and 27 patients having intermediate or high levels of *nm23* expression

The data in the present study suggest it is unlikely that the *nm23* gene is a metastasis-suppressor gene in human renal cell carcinoma. The *nm23* gene seems to have no role in the regulation of tumor progression and metastatic ability in renal cell carcinoma but might be an early genetic event in the evolution of the tumor.

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