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Original Paper

nm23 Protein Expression in Larynx Cancer and the Relationship with Metastasis

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The *nm23* gene, which encodes nucleoside diphosphate (NDP) kinase, is proposed as a metastatic suppressor gene and has been demonstrated to correlate inversely with metastatic potential in several tumours. To elucidate the role of *nm23* in larynx carcinomas, we examined using immunohistochemistry the expression of the nm23 protein in matched sets of primary tumours and metastatic lymph nodes. nm23 Protein was expressed in all the carcinomas as well as in non-neoplastic larynx mucosa. Overexpression of nm23 protein was found in the majority of primary tumours compared with corresponding normal mucosa, while decreased expression was associated with poor differentiation and distant metastasis and/or recurrence. No significant difference in age, sex and stage was found between primary tumours with high and low nm23 protein expression. These results suggest that decreased nm23 protein expression may play a role in metastasis and/or recurrence in larynx cancer and therefore could be used as a prognostic factor. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

TUMOUR METASTASIS is the principal cause of death for cancer patients. It is thought to be a complex process requiring cell motility, invasion, angiogenesis and avoidance of host immune responses.

The *nm23* gene was first identified on the basis of its reduced expression in highly metastatic murine melanoma (K-1735 TK) cells. The level of *nm23* mRNA was higher in the cells with low metastatic potential [1]. The transfection of *nm23* cDNA into these highly metastatic cells resulted in a reduced incidence of primary tumour formation and a significant reduction in metastatic potential [2]. Subsequently, two distinct human *nm23* genes were isolated as *nm23H1* and *H2*, both of which encode proteins of approximately 17 kDa that exhibit a 90% amino acid sequence identity [3]. Recently, a third gene, *DR-nm23*, which has approximately 70% sequence similarity to the putative metastatic suppressor genes, *nm23-H1* and *nm23-H2*, has been identified [4]. It has been shown that *nm23 H1* and *H2* are identical to human nucleotide diphosphate (NDP) kinase-A and -B, respectively [5].

nm23 expression at either mRNA or protein level is differentially expressed in human breast carcinomas and tumour cell *nm23* expression is associated with good prognosis and a lack of lymph node metastasis [6–8]. Allelic deletion of *nm23-H1* has been correlated with distant metastasis of colorectal carcinomas [9]. Reduced expression of nm23 protein in colorectal carcinomas is associated with tumour stage and distant metastasis [10].

However, there are few reports on the relationship between *nm23* expression and metastatic potential in tumours other than breast and colorectal carcinoma, and there has been no report on *nm23* in human larynx cancer.

In this study, we examined nm23 protein expression in primary laryngeal epidermoid carcinoma and lymph node metastasis by immunohistochemistry and investigated the relationship between nm23 protein expression and clinical and histopathological markers.

MATERIALS AND METHODS

Specimens

Surgically removed laryngeal tumours and corresponding normal mucosa stored as paraffin-embedded tissue blocks

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between 1978 and 1993 were obtained from the Departments of Otolaryngology and Pathology, Hacettepe University, Faculty of Medicine. The patients were selected randomly. Of the 86 patients, 78 were men and 8 were women; their ages ranged from 31 to 75 years (mean 52.6 years).

The tumours were staged according to the American Joint Committee on Cancer Criteria [11]: stage I, 6, stage II, 20, stage III, 42, and stage IV, 18. Histological type, differentiation and lymph node metastasis in the neck were examined by haematoxylin and eosin (H&E) stained 6 μm sections prepared from formalin fixed, paraffin-embedded tissue blocks.

Immunohistochemical staining

A modification of the immunoglobulin enzyme-bridge technique (ABC method) was used on formalin-fixed, paraffin-embedded sections of 86 specimens. Briefly, sections (6 μm thick) from each tissue block were deparaffinised, dehydrated, and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol. The sections were incubated with an anti-nm23 antibody for an hour, biotinylated antirabbit antiserum (Histostain-SP kit, Zymed lab. Inc., San Fransisco, California, U.S.A.), and avidin–biotin peroxidase complex (vectastain ABC kit, Vector) according to the instructions of the supplier. The peroxidase reaction was revealed by hydrogen peroxide in the presence of diaminobenzidine. Sections were counterstained with haematoxylin.

Immunoreactivity was graded as low and high nm23 expression according to number of cells stained and the intensity of the reaction in individual cells by two independent microscopic observations. Low expression was defined as almost no positive cells or small numbers of tumour cells showing positive immunoreactivity. High expression was defined as a large number of cells showing moderate and/or strong immunoreactivity.

Anti-nm23 polyclonal antibody was raised against a synthetic oligopeptide recognising the internal hydrophilic portion of the protein which lies between bases corresponding to 354 and 404 of human *nm23* cDNA (peptide 11) [10, 12]. The antibody recognises products from both *nm23-H1* and *-H2* genes, because the sequences corresponding to that of synthetic antigenic peptide 11 are identical in nm23-H1 and -H2 [5]. The specificity of the immunoreaction was confirmed by a pre-incubation step with the anti-nm23 antibody using an excess of the antigenic oligopeptide (peptide 11) of nm23. A negative control was incubated without primary antibody, while a colon carcinoma section of known positive immunoreactivity was used as the positive control [10]. Statistical comparisons between groups were performed by the chi-square, Fisher's exact chi-square or Student's *t*-test.

RESULTS

Immunocytochemistry

All tumours and normal samples expressed nm23, but increased expression over levels in non-neoplastic tissue was seen in 70% of carcinomas (Figure 1). Of the 86 primary tumours, 54 (63%) showed high nm23 expression while 32 (37%) showed low expression. A representative illustration of nm23 expression in a primary tumour and its corresponding metastatic lymph node is shown in Figure 2.

Correlation with clinicopathological parameters

The correlation of nm23 expression of the primary tumours with clinicopathological parameters is shown in Table 1. There was no association with age or stage of disease, but there was a significant correlation between low nm23 expression and poor differentiation ($P < 0.05$, chi-square). However, while there was also a correlation between low nm23 expression and recurrence and/or the presence of distant metastases ($P < 0.05$, chi-square), there was no association between nm23 expression in the primary tumour and the occurrence of lymph node metastases in the neck. Of the 42 primaries without neck lymph node metastases (which showed a heterogeneous staining pattern), 30 (71%) had high expression and 12 (29%) had low expression, whilst of the 44 patients with neck lymph node metastases, 24 (55%) of the primaries had high nm23 expression and 20 (45%) had low expression.

Table 2 shows the correlation between nm23 expression in the 44 primary tumours and their corresponding lymph node metastasis. All 20 lymph node metastasis from the primaries with low nm23 expression also had low nm23 expression, whilst in contrast, only 5/24 (21%) of the lymph node metastasis from primaries with high nm23 expression also had high nm23 expression. The other 19/24 corresponding lymph node metastasis had low nm23 expression ($P < 0.05$).

DISCUSSION

Expression of the *nm23* gene either at the mRNA or protein level has been reported to be inversely related to the metastatic potential of human cancers such as breast and colorectal

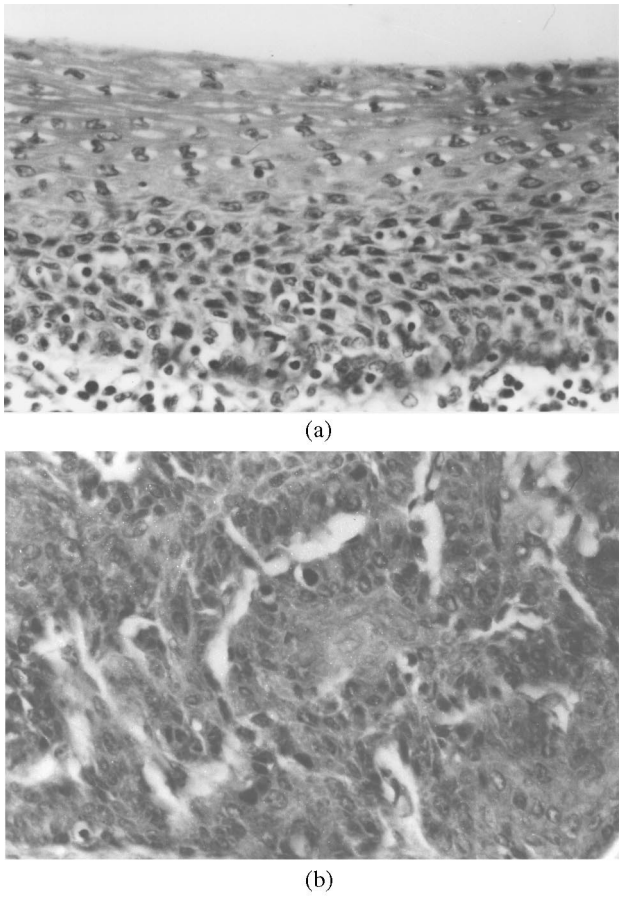

Figure 1. nm23 immunoreactivity of the normal mucosa was weaker than tumour tissue (immunoperoxidase, 230x). (a) Normal mucosa; (b) tumour tissue.

carcinoma. In our study, all tumours and normal samples expressed nm23 protein, but increased expression was found in 70% of tumours compared with the corresponding normal mucosa. Similar findings have been reported in other types of cancers such as breast, colorectal and gastric carcinoma [8, 10, 12].

Although nm23 protein expression was reduced in primary tumours with neck metastasis compared with non-metastatic carcinomas, there was no statistical difference. However, we observed that nm23 protein expression in metastatic lymph nodes was clearly lower than their corresponding primary tumours. Although these findings do not clarify the biological action of the *nm23* gene, the immunohistochemical verification of the heterogeneous existence of the *nm23* gene within a given primary tumour and the reduced level of nm23 protein expression in metastatic lymph nodes compared to their corresponding primary tumours suggests that metastatic tumour cells originate from, and are mainly composed of the cells with low nm23 protein expression. These data suggest that the *nm23* gene plays a role in the metastatic process.

While a relationship between nm23 gene expression and tumour stage was found in lung cancer [15] and hepatocellular carcinoma [16], we could not find any relationship between the tumour stage and *nm23* protein expression, confirming the results of Ayhan and associates in colorectal carcinoma [10]. However, the poorly differentiated carcinomas showed reduced expression of nm23 protein compared with well- and moderately differentiated carcinomas. These findings show that one of the signs of metastatic potential, that is, poor differentiation, correlates with reduced nm23 protein expression.

The role of nm23 in distant metastasis is yet unknown. However, Ayhan and associates demonstrated an inverse correlation between nm23 protein expression of primary tumours and their corresponding metastasis in colorectal carcinoma [10]. They also found heterogeneous immunostaining within a primary tumour which could suggest a different metastatic capacity for the various component cells. Their findings showed that nm23 immunoreactivity of distant metastasis was less than their corresponding primary sites.

Nakayama and associates reported that 71% of gastric carcinomas showed weaker nm23 immunoreactivity in liver

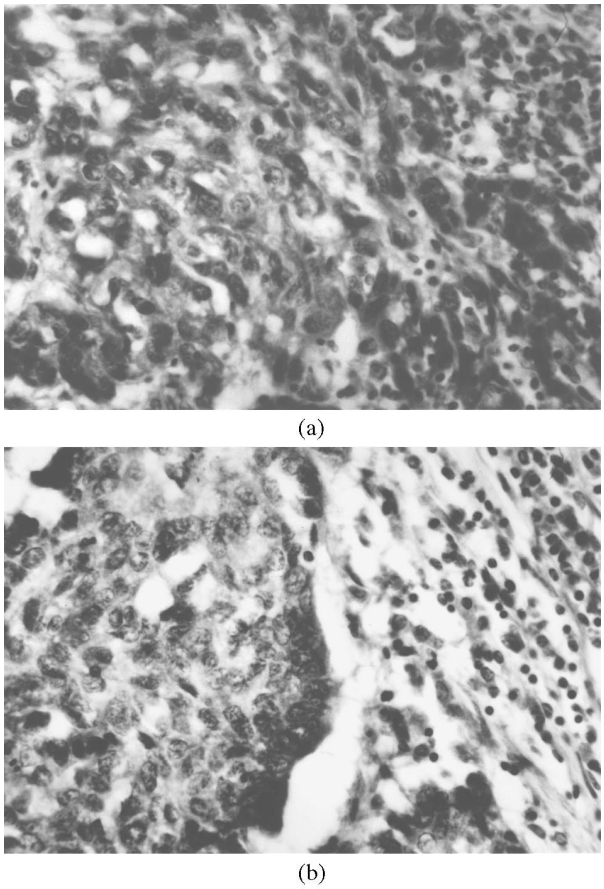

Figure 2. nm23 protein expression in a primary tumour and its corresponding metastatic lymph node. (a) Primary tumour. (b) Metastatic lymph node. nm23 immunoreactivity in the primary tumour was much stronger than that in the metastatic lymph node. nm23 immunoreactivity was observed in the primary lesion both in the cytoplasm and nucleus of the tumour cells (immunoperoxidase, 460x).

Table 1. *Relationship between nm23 protein expression of primary tumours and clinicopathological features*

Variable	No. of patients		Significance
	Low	High	
Age	52.53†	52.72†	N.S.
Sex			
Male ($n=78$)	30 (38%)	48 (62%)	
Female ($n=8$)	2 (25%)	6 (75%)	N.D.
Lymph node metastasis			
No ($n=42$)	12 (29%)	30 (71%)	
Yes ($n=44$)	20 (45%)	24 (55%)	N.S.
Stage			
1 ($n=6$)	1 (17%)	5 (83%)	
2 ($n=20$)	9 (45%)	11 (55%)	
3 ($n=42$)	13 (31%)	29 (69%)	
4 ($n=18$)	9 (50%)	9 (50%)	N.S.
Differentiation			
well ($n=30$)	5 (17%)	25 (83%)	
moderate ($n=29$)	7 (24%)	22 (76%)	
poor ($n=27$)	20 (74%)	7 (26%)	$P<0.05$
Recurrence and/or distant metastasis*			
No ($n=54$)	17 (31%)	37 (69%)	
Yes ($n=16$)	10 (63%)	6 (38%)	$P<0.05$

*16 patients were lost to follow-up. Median follow-up for the other 70 patients was 31 months (range 5–110). †Mean age for low and high nm23 expression.
N.S. = non-significant; N.D. = not done.

Table 2. *Comparison of nm23 protein expression of metastatic primary tumours with corresponding lymph node metastasis* (n = 44)

	Metastatic lymph node	
	Low ($n=39$)	High ($n=5$)
Primary tumours		
low ($n=20$)	20 (100%)	0
high ($n=24$)	19 (79%)	5 (21%)
total ($n=44$)	39 (88%)	5 (12%)

Statistical analysis: $P<0.05$.

metastasis than in the primary tumour [12]. Their findings of heterogeneous existence of nm23 within a given primary tumour and the reduced level of nm23 protein in distant metastatic foci suggested that metastatic tumour cells originate from cells with low nm23 protein expression. In the present study,the primary tumours with high nm23 protein expression showed less distant metastasis and/or recurrences compared with low nm23 protein expressed tumours, whilst the primary tumours with low nm23 expression revealed a more aggressive pattern. These data suggest that the *nm23* gene may also play a role in distant metastasis or recurrences and could be used as a prognostic factor.

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