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Relationship of Nm23 Expression to Proliferation and Prognosis in Malignant Melanomas of the Oral Cavity

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Abstract. *Twenty-nine cases of oral melanomas were investigated for nm23 and Ki67 antigen expression, as well as for the fraction of tumour cells in S-phase, using immunohistochemical techniques and DNA cytophotometry. Nm23 expression was significantly reduced and Ki67 antigen expression increased in primary tumours with either lymph node or organ metastases in comparison to tumours without metastases. The percentages of Ki67 immunoreactive tumour cells and cells in S-phase correlated positively with each other and negatively with the percentage of nm23-expressing cells. These data argue against a significant growth stimulatory function of the nm23H1 gene product nucleoside diphopshate kinase in the progression of oral melanomas. The functional relevance of nm23 in relation to increased proliferation and metastatic spread is discussed.*

An important role of nm23 in DNA replication and cellular proliferation has been suggested in several studies (1-4). These reports documented a correlation between increased nm23 expression and proliferation. Increased nm23 level was found in proliferating peripheral blood lymphocytes (5). However, the main role of this gene in some types of malignancy apparently is to interact with the metastasic process and, therefore, a significant correlation between low nm23 level and high metastatic potential was found in several tumours (6-10). The probable prognostic significance of nm23 in cutaneous melanoma was shown by our group in a former study (11). The main objective of this study was to investigate the expression of nm23 in a series of melanomas of the oral cavity and sinus maxillaris in order to define the prognostic significance of this marker. Additionally, the expression of nm23 was related to parameters of proliferative activity, which have been shown to be factors of poor prognosis for oral melanoma patients (12).

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Materials and Methods

The material investigated consisted of 29 oral melanoma cases located as follows: cheek 8, maxilla 9, mandible 4, sinus maxillaris 6 and palate 2. There were 8 female and 21 male patients. The age of patients ranged between 41 and 87 years, averaging 67 years. The patients were treated surgically in Krakow, Poland and in Göttingen, Germany. Twenty-three patients had involvement of lymph nodes and 12 of them also of distant organs in metastatic disease.

Samples were processed routinely using formalin fixation and were embedded in paraffin. To detect nm23 expression, the mouse monoclonal antibody clone NM301 (Pharmingen, Heidelberg, Germany), which recognizes the human nm23H1 protein, was applied. To detect Ki67 expression, MIB1 antibody from Dianova (Hamburg, Germany) was used. Microwave pretreatment (2x5 min in citrate buffer pH=2) was necessary to unmask antigens in reaction for nm23 and Ki67. The nm23 reaction product was demonstrated using the Novostatin Super ABC kit (Novocastra, Newcastle-upon-Tyne, UK) and DAB as chromogen. To detect Ki67 antigen, the APAAP complex (Quartet, Berlin, Germany) and new fuchsin as chromogen were applied.

S-phase was evaluated on Feulgen-stained sections, by CAS200 image analyzer and QDA software package. Three-μm-thick histological sections were deparaffinized and treated for 60 min with 5N HCl. The histological sections were then stained with the Feulgen kit according to the manufacturer's instructions (CAS

Table I. *Relationship between marker positivity and metastatic spread of oral melanomas.*

Group of patients	Ratio of positive cases		
	Nm23	Ki67	S-phase
N=0 M=0	6/6	6/6	5/6
N>0, M=0	9/11	11/11	11/11
N>0, M>0	4/12	12/12	12/12

Table II. *Relationship between marker expression and metastatic spread of oral melanomas.*

Group of patients	Nm 23 (%)		Ki67 (%)		S-phase (%)	
	Range	Mean	Range	Mean	Range	Mean
N=0 M=0	61-100	74.0	4-29	18.0	0-12	7.0
N>0, M=0	0-42	25.9	8-79	47.3	1-27	11.2
N>0, M>0	0-6	2.8	12-95	68.6	9-43	26.2

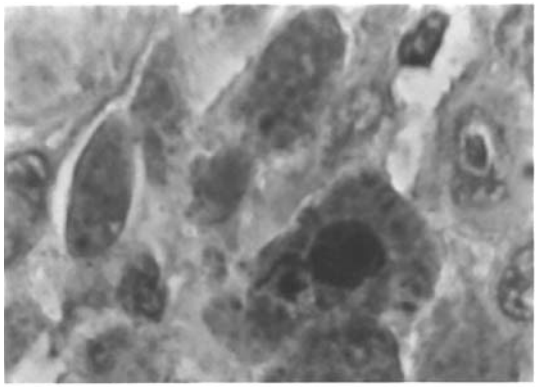

Figure 1. *Nm23 cytoplasmic positivity in oral melanoma (x400).*

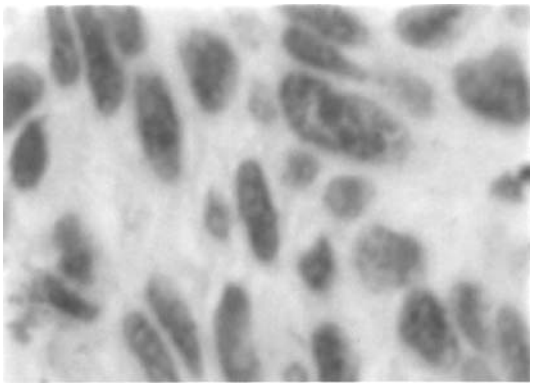

Figure 2. *Ki67 nuclear positivity in oral melanoma (x400).*

DNA dye kit for cell analysis system; Becton Dickinson, Hamburg, Germany). Following dehydration in an ascending alcohol series, the sections were transferred in xylol and covered with a synthetic medium. Feulgen-stained rat hepatocytes were used as control cells for calibration of the CAS200 system. S-phase evaluation involved the measurement of 200 tumour cells. The results were expressed as percentages of positive cells (=indices): nm23 index, Ki67 index, S-phase index. In order to compare the group of patients with and without metastases, the Mann-Whitney *U*-test was applied. The relationship between markers was defined applying the correlation coefficient according to Spearman.

Results

Group of patients without metastases. In the group of patients without metastases (n=6), all cases were nm23-positive (Table I, Figure 1). The nm23 index ranged between 61 and 100%, averaging 74%. Ki67 expression was also found in 6 cases without any metastases. The Ki67 index ranged between 4 and 29%, averaging 18%. The S-phase was respectively lower and did not exceed 12% (mean 7%) (Table II).

Group of patients only with lymph nodes involvement. In the group of patients with lymph nodes involvement (n=11), nm23 expression was found in 9/11 cases. The nm23 index ranged between 0 and 42%, averaging 25.9%. Ki67 expression was observed in all cases belonging to this group (Figure 2). The Ki67 index oscillated between 8 and 79%, being on average 47.3%. The S-phase index was lower than the Ki67 index and ranged between 1 and 27%.

Group of patients with lymph nodes and organs involvement. Nm23 positivity was found in 4/12 primary melanomas with the presence of lymphatic and distant metastases. The nm23 index did not exceed 6%. The Ki67 antigen was found in all melanomas with lymph nodes and organ involvement. The positivity for Ki67 ranged from 12 to 95%, averaging 68.6%. The S-phase was higher than in the formerly described groups and oscillated between 9 and 43%, averaging 26.2% (Figure 3).

Differences between groups investigated. Nm23 expression differed highly significantly between tumours with and without lymph nodes and organs involvement ($p<0.01$). The Ki67 index differed significantly when melanomas with and without metastases were compared ($p<0.05$) and highly significantly when melanomas without any metastases were compared with ones which had already formed organ metastases ($p<0.01$). Differences concerning the S-phase with respect to lymphatic and distant metastatic spread did not reach statistical significance.

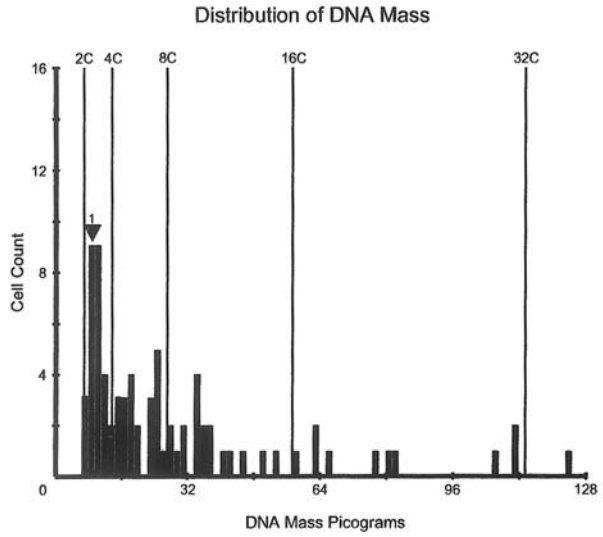

Figure 3. *DNA histogram of a case of primary oral melanoma with lymph node and distant metastases and high S-phase measured by image analysis system CAS200.*

Relation between parameters investigated. All 3 parameters investigated correlated significantly with each other. However, the correlation of S-phase-Ki67 antigen was more significant ($p<0.01$) than the relationships between nm23 and both proliferation parameters ($p<0.05$) (Table III).

Discussion

In this study, a decreased expression of nm23 along with oral melanoma metastatic progression was found. Cases in which distant metastases were known had significantly lower nm23 levels than the initial stadia of melanoma disease. The described relationship is in accordance with many reports about a decreased nm23 level along with metastatic progression in various non-melanocytic tumour types (13-16). This phenomenon was confirmed by previous studies on nm23 expression in cutaneous melanomas (11, 17, 18). Clear statistical differences between cases with and without metastases, gained with the help of a nonexpensive detection system, allow us to postulate the practical application of these markers (nm23, Ki67) to screen patients for antimetastatic therapy.

In this study, the prognostic significance of the Ki67 antigen was also shown. This marker is known to be expressed in G1-, S-, G2- and M-phases of the cell cycle (19, 20). The expression of Ki67 antigen not only in the S-phase, but also in G1-, M- and G2-phases, explains the lower level of S-phase in comparison with the Ki67 indices. The strong correlation between both proliferation indices is obviously due to their cell cycle specificity.

Table III. *Correlation between parameters investigated.*

Parameter	Nm23	Ki67	S-phase
Nm23	X	*	*
Ki67	*	X	**
S-phase	*	**	X

*significance $p<0.05$
**significance $p<0.01$

In addition, an inverse correlation between nm23 and Ki67 expressions was demonstrated in this study, which, to our knowledge, has not been checked previously in malignant melanomas. This statistical relationship argues against an important growth stimulatory function of the nm23 gene-encoded protein nucleoside diphosphate in the progression of oral malignant melanoma. The functional relationship of nm23 expression to the mechanisms of metastatic spread and proliferation in malignant melanomas is still unclear. Recent studies have stressed the importance of the expression of cytoskeletal elements for the invasive and metastatic behaviour of melanoma cells (21). Nm23 also affects the polymerisation of cytoskeletal elements and this may be the mode of action by which it modulates signal transduction and thereby confers invasive properties (22). Alternatively,one should consider the possibility that other genes, such as the murine 18A2/mts1 and its human homologue h-mts1, are co-regulated with the nm23 suppressor gene (22, 23), which may explain both increased proliferation and metastatic spread in the group of nm23-negative melanomas.

The clinical outcome of some cases demonstrated in this study have been published previously (24). Most investigators emphasize that early diagnosis and radical surgery are the decisive factors in the generally poor prognosis of oral malignant melanomas (24, 27).

Unfortunately, the majority of the tumours investigated in this study were treated at a late stage and, therefore, the demonstration of nm23 expression in a broad spectrum of oral melanoma progression was not possible in this study.

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