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Advances in Brief

Levels of *nm23* Messenger RNA in Metastatic Malignant Melanomas: Inverse Correlation to Disease Progression[1]

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Abstract

Data obtained in experimental murine tumors and in clinical specimens of human breast cancer have suggested that the *nm23* gene may function as a metastasis suppressor gene. In this report we examined the *nm23* mRNA level in tumor tissue obtained from distant metastases in 33 patients with malignant melanoma. The gene was differentially expressed in the tumors with a 20-fold range in hybridization intensities. The levels of *nm23* mRNA in benign nevi obtained from 12 of the 33 patients were relatively low, with a mean value of 17% of that in the melanomas. In attempts to relate the level of *nm23* expression in the tumor metastases to progression of the disease, the time from biopsy of the primary tumor to the appearance of metastases was used as a clinical end point. It was found that patients developing metastases during the first 2 years after diagnosis had significantly lower levels of tumor *nm23* expression (56% of the mean value) compared to patients with less aggressive disease (164%) ($P < 0.0004$). In concordance with previous data the association found here between low levels of *nm23* mRNA and the malignant potential of melanomas suggests that the *nm23* gene may be implicated in the mechanism of disease progression in some types of human cancer.

Introduction

An overt cancer metastasis is the result of a cascade of sequential steps. The search for genes that may be responsible for promoting or suppressing the metastatic process has led to identification of several interesting candidates (1–4). The *nm23* gene, suggested to represent a new class of metastasis associated genes (4–6), was originally identified in differential colony hybridization experiments involving murine K-1735 melanoma sublines with low and high metastatic potential (4). The expression of the *nm23* gene has been shown to be inversely related to the metastatic potential of K-1735 cell lines and *N*-nitrosomethylurea induced rat carcinomas (4), as well as of cells transformed by the adenovirus 2 *E1a* gene and of c-Ha-*ras* transformed rat fibroblasts (5). Moreover, transfection of the cloned human homologue gene *nm23-H1* cDNA[3] into highly metastatic K-1735 TK cells, significantly reduced their metastatic potential, independent of the effect on tumor cell growth rate (6).

In human tumors, the level of *nm23* mRNA has been found to be markedly reduced in primary, infiltrating ductal breast cancers with metastases in regional lymph nodes present at diagnosis (7). In a separate study, low *nm23* expression in breast tumors correlated with both decreased disease free and overall survival (8). However, Haut *et al.* (9) found increased *nm23* gene expression in neoplastic colon tissue compared with the levels in morphologically normal colon mucosa from the same individuals. Moreover, in a study on neuroblastoma Hailat *et al.* (10) found that high levels of the *nm23* protein were associated with advanced stage of the disease.

On the background of the seemingly contradictory results obtained in the different types of tumors, and since the *nm23* gene originally was cloned from murine melanoma cells, we examined *nm23* expression in human melanoma metastases. The results demonstrate an association between *nm23* expression in the melanomas and a clinical parameter of tumor progression.

Materials and Methods

Specimens. Tumor tissue and peripheral blood were obtained from 33 patients with metastatic malignant melanoma, and in 12 of these cases a benign nevus was also biopsied. In addition, benign nevi from 10 healthy volunteers were sampled. Immediately upon surgery, the tissues were frozen in liquid nitrogen and thereafter stored at −135°C.

Southern Blot Analysis. Genomic DNA from melanoma tissues and peripheral blood cells were isolated by standard methods (11). Aliquots (10 μg) of DNA were digested with an appropriate restriction enzyme, separated on 0.8% agarose gels, and transferred onto Hybond N^+ membranes (Amersham), according to the manufacturer's manual.

After baking for 2 h at 80°C and subsequent UV cross-linking, the blots were hybridized with DNA probes labeled with ^{32}P by the random primer technique (12). The hybridization was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM sodium EDTA at 65°C for 16 h as described by Church and Gilbert (13). After hybridization, the membranes were washed three times for 20 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS. For multiple hybridizations, the bound probe was removed by incubating the filters twice for 5 min in 0.1 × standard saline-citrate (3.0 M sodium chloride-0.3 M sodium citrate, pH 7.0) and 0.1% SDS at 95–100°C.

Probes and Restriction Enzymes. To detect allelic deletions on chromosome 17, the following probes and restriction enzymes were used: The 900-base pair *Bam*HI fragment of *nm23-H1* cDNA, *Bgl*II (14); pCMM86 (*D17S74*), *Taq*I (15); pHF12-2 (*D17S1*), *Msp*I (16), and pBHp53, *Bam*HI (17).

Northern Blot Analysis. Total RNA was isolated from malignant and benign tissues by the guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (18) or the guanidinium thiocyanate-CsCl method described by Maniatis *et al.* (11). Samples of 5 μg of total RNA were resolved by electrophoresis on 1% agarose-formaldehyde gels (11) and blotted onto Hybond N^+ membranes according to the manufacturer's manual. The filters were baked, hybridized to the *nm23*-H1 cDNA probe and stripped as described for Southern blot analysis. To correct for the uneven amount of RNA loaded in each lane, the filters were rehybridized to a kinase-labeled (11) oligonucleotide (19 bases) specific for human 18S rRNA. The level of *nm23* mRNA was adjusted relative to the amount of 18S rRNA after scanning of the autoradiograms in a Molecular Dynamics Computing Densitometer.

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[3] The abbreviations used are: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; RFLP, restriction fragment length polymorphism; LOH, loss of heterozygosity; NDP, nucleoside diphosphate.

Results

***nm23* Expression.** The tumor tissue examined was obtained from distant metastases in 33 patients with malignant melanoma, in which the corresponding primary tumors were of different histological subtypes and localization (Table 1). When total RNA prepared from the metastases and from biopsies of benign nevi in 10 healthy volunteers was analyzed on Northern blots, it was found that the *nm23-H1* gene was expressed at highly variable levels in the malignant tumors (Fig. 1). Thus, for the melanomas, a more than 20-fold range in hybridization intensities was demonstrated by densitometry (not shown), whereas the levels in benign nevi were lower and less variable. The mean level of *nm23* expression in the melanomas was 5-fold higher than in the benign nevi.

That *nm23* expression is elevated in malignant compared to benign tumors of melanocytic origin was further confirmed when the expression level in paired samples from tumor and nevus in the same patient was compared (Fig. 2). The relative levels of *nm23* mRNA, after adjusting for uneven amounts of RNA loaded on the gel, are illustrated in Fig. 3. The mean value for the hybridization intensity in the nevi was only 17% of that found in the melanomas. In 10 of the 12 matched pairs, the gene expression level in the tumors greatly exceeded that of the corresponding benign nevus. In the last 2 cases the relative tumor levels were low, both in the melanomas (less than 15%), and in the nevi (5%).

Relationship between *nm23* Expression and Disease Progression. Attempts were made to correlate the expression of *nm23* with disease progression parameters for the patients. The time from biopsy of the primary tumor to the development of the distant metastases examined was used as a clinical end point. Therefore, the three cases in which the primary tumor was unknown and the two in which the biopsies were taken from local recurrences had to be excluded from this evaluation. The remaining 28 cases could be divided into 2 equally sized groups (Table 2), one including those patients who developed metastases within 2 years of follow-up (mean, 9 months), the other those with a relapse free period of more than 2 years (mean, 64 months).

Table 1 *Tumor characteristics of 20 male and 13 female melanoma patients*[a]

Localization of primary tumor					Histology			
Head and neck	Extremity							
	Truncus	Upper	Lower	NS[b]	Nodular	Superficial	Other	NS
3	13	3	11	3	11	14	5	3

[a] Mean age, 58 years (range, 26–85).
[b] NS, not specified.

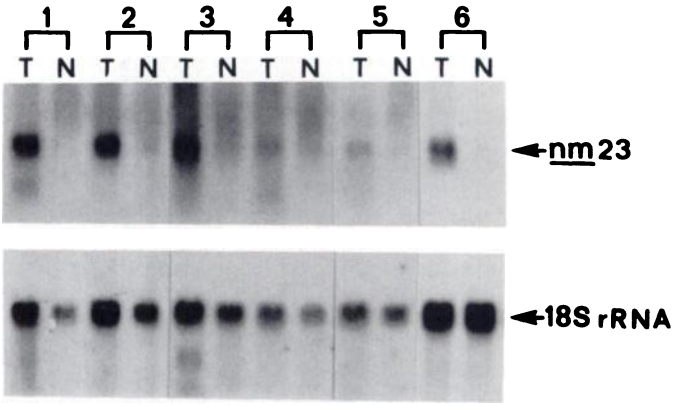

Fig. 2. Northern blot analysis showing the *nm23* mRNA level in six matched pairs of tumor and benign nevus from the same patient. Total RNA from the tumor specimens (*T*) and from the benign nevi (*N*) was hybridized to the *Bam*HI fragment of *nm23-H1* cDNA (*top*) and to a 18S rRNA probe as a control (*bottom*).

The level of *nm23* mRNA in the tumors was expressed as a percentage of the mean *nm23* level. As shown in Fig. 4 and Table 2, patients developing metastases during the first 2 years had significantly lower levels of tumor *nm23* expression (56%) compared to patients with less aggressive disease (164%) ($P < 0.0004$; Fisher's one tailed test). It should be noted, however, that in 2 of 14 cases in the group with aggressive tumors the gene expression levels were relatively high (133 and 134%), whereas for 2 of 14 patients with late relapses the levels were below (50 and 97%) the mean (Fig. 4; Table 2).

In concordance with this, the primary tumor was thicker (3.4 mm) in the group of patients with low compared to the group (2.6 mm) with high levels of *nm23* mRNA, although this difference was not significant. No difference in *nm23* expression was observed between different histological melanoma subgroups (not shown).

RFLP Studies. In attempts to investigate whether differences in tumor mRNA expression levels could be related to allelic deletions of the *nm23*-H1 gene, DNA from 24 matched pairs of tumor tissue and peripheral blood cells were studied for LOH in the relevant area on chromosome 17q. Thirteen (54%) of the tumors were informative for the *nm23-H1* locus (17q21), and of these, only two (8%) exhibited a deletion of one *nm23-H1* allele (data not shown). In both tumors, LOH was also observed for *D17S74*, a locus close to *nm23* on the chromosome (15). One tumor that was not informative on the *nm23-H1* locus showed LOH on *D17S74, D17S1* (17p13), and pBHp53 (17p13.1), presumedly indicating that most of one chromosome 17 was lost, including the *nm23-H1* allele. It is noteworthy that these three cases all belonged to the group of

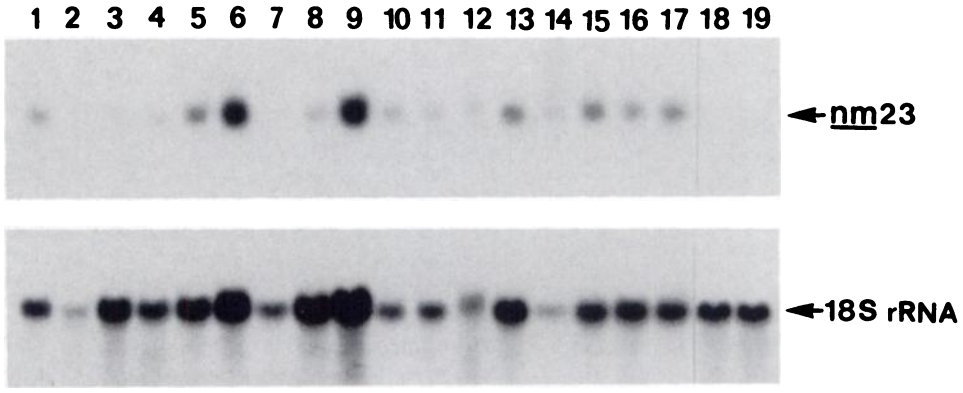

Fig. 1. Representative Northern blot analysis demonstrating the *nm23* mRNA level in human melanomas (*Lanes 1–17*) and in benign nevi from healthy individuals (*Lanes 18* and *19*). Total RNA was hybridized to the 900-base pair *Bam*HI fragment of *nm23-H1* cDNA (*top*), and as a control to a 18S rRNA probe (*bottom*) as described in "Materials and Methods."

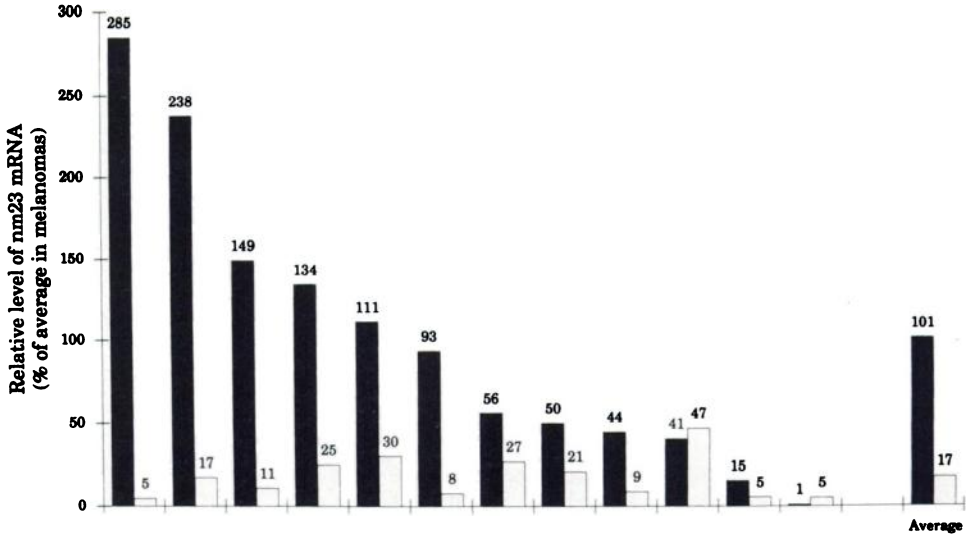

Fig. 3. Quantitation of the relative *nm23* mRNA level in 12 matched pairs of tumor (■) and benign nevus (□) from the same patients. The level of *nm23* mRNA was assessed by densitometry scanning and in each case adjusted according to the amount of 18S rRNA. The results are given as percentage of the average *nm23* expression level in the melanomas.

Table 2 *Relationship between the levels of nm23 gene expression and disease progression in melanoma patients*

Relative levels of *nm23* mRNA (% of average)	No. of patients developing distant metastasis after		
	<24 (mean 8) mo	>24 (mean 64) mo	Total
Low (<100)	12	2	14
High (>100)	2	12	14

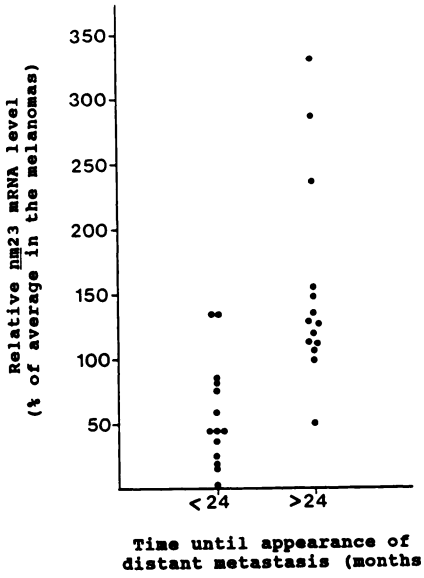

Fig. 4. Relationship between tumor *nm23* mRNA levels and disease progression in patients with malignant melanoma. The relative level of *nm23* expression in melanoma metastases, determined as described in "Materials and Methods," was plotted against time from diagnosis until appearance of metastases. The 28 examined cases were divided into 2 equally sized groups, one including those developing metastasis within the first 24 months, the other those that had a longer relapse free period.

patients with the lowest *nm23* mRNA expression and the shortest relapse free period.

Discussion

Evidence has been provided suggesting that *nm23* may act as a metastasis suppressor gene (7, 8, 19, 20). Thus, in primary breast cancer, low tumor levels of *nm23* mRNA have been associated with clinical parameters indicating a good prognosis (7, 8). Moreover, in this disease the degree of immunohistochemical staining of tumor specimens with antibodies directed against the *nm23* product, or against the protein of the homologous NDP kinase gene, has been found to be inversely correlated to patient survival (19, 20). On the other hand, studies in colorectal carcinoma, in neuroblastoma, and on tissue specimens from several other tumor types (9, 10, 21) did not reveal a similar relationship between *nm23* expression and the aggressiveness of the disease. These apparently contradictory results might indicate that *nm23* can function as a suppressor gene in some types of cancer but can be associated with tumor aggressiveness in others.

The present data suggest that expression of the *nm23* gene may be related to disease progression in patients with malignant melanoma. We observed that the *nm23* gene was differentially expressed in biopsied material from metastatic tumor lesions and that the mRNA level was significantly lower in tumors from patients with a short period from primary diagnosis until the metastases developed than from those with more prolonged relapse free intervals. It should be noted that no relationship between the level of expression and the size and localization of the primary tumor was found. Moreover, the variation in *nm23* expression was similar in nodular, superficial, and other melanoma subtypes (data not shown).

In 12 of the 33 cases we were able to compare *nm23* expression in melanoma metastasis with that found in benign nevi from the same patients. In contrast to what might have been expected, the expression levels were much lower in the benign tumors. These results bear some resemblance to data obtained by Haut *et al.* (9) where *nm23* expression was higher in metastatic colon cancer than in normal colon mucosa, although similar to that found in benign polyps. Obviously, the mechanism of the suggested involvement of the *nm23* gene in tumor progression may be rather complex. In relation to the low *nm23* expression levels found here in nevi compared to in melanomas, it is of interest that Okabe-Kado *et al.* (22) have shown that the *nm23* protein is a candidate suppressor protein for differentiation of leukemic cells. It may be speculated that if the *nm23* protein should have a similar function in cells of melanocytic origin, the already differentiated cells in the benign nevi would not need a high *nm23* expression.

Recent data obtained in neuroblastoma demonstrated that genomic amplification of the *nm23* gene was associated with increased tumor RNA expression and reduced patient disease

free survival.[4] Since evidence for *nm23* mutation was found, it was concluded that molecular alterations of the *nm23* gene other than its reduced expression can be associated with tumor aggressiveness. In colorectal carcinoma, Cohn *et al.* (23) found a correlation between *nm23-H1* allelic deletions and the development of distant metastases. Although our RFLP data do not permit any definite conclusions, allelic deletions were found in 3 melanomas, all belonging to the group of 14 patients with short relapse free periods. Moreover, it is conceivable that *nm23* aberrations not detectable by RFLP studies might be present in malignant melanomas. It might be considered that allelic loss involving *nm23-H1* could result in altered activity of the gene product. Importantly, however, one cannot rule out the possibility that it is not only *nm23* itself but also a gene closely linked to it that is a target for allelic deletion (23).

Although the precise biological function of the *nm23-H1* gene is not known, its product has recently been shown to be identical to the NDP kinase A in human erythrocytes (24). In addition, *nm23* is highly homologous to the *awd* gene of *Drosophila*, a gene which also encodes a NDP kinase, and in which mutations or decreased expression induce developmental abnormalities (14, 25). NDP kinases are known to participate in microtubule assembly and disassembly (26). Furthermore it has been postulated that the NDP kinases may be involved in signal transduction through G-proteins (27), but recent data strongly oppose the hypothesis that NDP kinases can directly activate the regulatory GTP-binding protein.[5]

The association between *nm23* mRNA expression and tumor aggressiveness found in this study may have implications for the understanding of mechanisms involved in melanoma progression. It will be important to study the regulation of *nm23* expression in melanoma tissue obtained at different stages of the disease and whether the gene might be heterogenously expressed within each tumor. Work has been initiated to study the distribution of the *nm23* protein in a variety of tumor specimens obtained from patients with malignant melanoma.

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