NM23 expression in metastasis of malignant melanoma is a predictive prognostic parameter correlated with survival

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Summary The management of patients presenting with metastatic malignant melanoma (MM) is hampered by the substantial variability in survival of these patients and the lack of prognostic markers. In the search for a reliable predictive parameter, we have investigated the expression of the nm23 gene, considered to be a major regulator of the metastatic process. We have analysed by Northern blot the nm23 mRNA level in tumour tissue obtained from metastases of 20 stage II and ten stage III patients with MM. Normal human tissues and benign naevi were simultaneously examined. The level of nm23 expression was highly heterogeneous in MM metastases, with a mean value which was higher than the mean level in normal tissues and naevi. Correlative study focused on the overall survival following resection of the metastasis in which nm23 Northern blot analysis was performed. Patients displaying higher nm23 expression in metastatic tissue (above the mean level) tended to have a longer survival than others (P = 0.08), and this difference was significant for patients presenting with isolated regional lymph node involvement (P = 0.035). The time from biopsy of the primary MM to the appearance of the first lymph node metastasis correlated with the nm23 mRNA level in this metastasis. The present study is not only in accordance with previous reports showing that the nm23 gene may be implicated in MM progression, but also suggests the reliable value of nm23 expression as a prognostic marker for patients presenting with metastatic MM.

Current methods to identify the aggressive potential of malignant melanoma (MM) are limited. Even after occurrence of regional lymph node metastasis, patients may either pursue an indolent clinical course or rapidly die. The search for reliable prognostic parameters therefore appears vitally important in order to ensure adequate therapy, especially for advanced MM stages which are candidates for non-surgical treatment.

The production of clinically relevant metastasis is triggered by a complex series of linked sequential steps, some being genetically regulated by transient or permanent alterations at the DNA or mRNA level. The nm23 gene is thought to play a major role in this network of triggering signals (Rosengard et al., 1989; Leone et al., 1991). This gene was identified by differential colony hybridisation between related low- and high-metastatic murine k-1735 melanoma cell lines, a tumour system which contains clonal populations with qualitative differences in metastatic capacity in syngeneic mice (Steeg et al., 1988). mRNA levels of the nm23-1 gene were found to be approximately 10-fold higher in low-metastatic potential clones than in highly metastatic clones (Steeg et al., 1988).

In human tumours, contradictory results were reported on nm23 gene expression. Reduced expression was found in primary, infiltrating ductal breast carcinomas with metastases in regional lymph nodes present at diagnosis (Bevilacqua et al., 1989; Hennessy et al., 1991). Low nm23 expression in breast tumours also correlated with decreased survival (Barnes et al., 1991). These findings, however, cannot be generalised since low nm23 expression does not clearly imply poor prognosis in other types of human tumours such as colorectal carcinoma or neuroblastoma (Cohn et al., 1991; Hailat et al., 1991; Haut et al., 1991). The prognostic value of the nm23 gene transcriptional activity in MM is suggested by the fact that this gene was originally cloned from murine melanoma cells, and also by some preliminary observations in human MM (Florenes et al., 1992). In this report, we have tried to investigate the significance of nm23 expression as a parameter for the practical management of advanced-stage MM.

Materials and methods

Tumour sampling

Tumoral tissue samples from 30 patients with MM were obtained through surgery. These patients were classified as stage II (regional lymph node involvement, n = 20) or stage III (distant lymph node involvement or visceral metastasis, n = 10). The histopathological characteristics of the primary cutaneous MM are detailed in Table I.

Each biopsy specimen was histologically identified as metastasis of MM involving lymph node in 25 cases, skin in four cases and liver on one case. A part of each fresh sample was stored in liquid nitrogen.

In addition, eight samples of human normal tissues (liver, breast, prostate, lymph node, spleen and ovary) as well as three benign naevi were analysed.

Northern blot analysis

Total RNA was isolated from frozen tissues by the guanidinium thiocyanate–cesium chloride method as previously described (Maniatis et al., 1982).

Integrity of each RNA sample was ensured by (i) electrophoresis of a 2 μg aliquot on denaturing agarose–formaldehyde gel; and (ii) reverse transcription and polymerase chain reaction (PCR) amplification of the human GAPDH gene, which is expressed in almost all types of tissues. Northern blots were performed by running 10 μg of RNA on denaturing gels and transferring onto Hybond nylon membranes as indicated by the manufacturer (Amersham, UK).

The filters were UV cross-linked and hybridised to the nm23-H1 cDNA probe (a 900 bp BamHI fragment from pNM23-H1 plasmid, kindly provided by Dr P.-S. Steeg, NCI, Bethesda, MD, USA). Filters were then stripped and rehybridised to a cDNA probe specific for human GAPDH to correct for the unequal amount of RNA loaded in each lane. The level of nm23 mRNA was adjusted relative to the amount of GAPDH RNA after densitometric scanning of the autoradiograms. GAPDH was chosen as an internal standard because this gene is resistant to transcriptional induction by various agents and is known to show a relatively constant expression among most tissues (Bosma & Kooistra, 1991; Zentella et al., 1991).

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**Statistical analysis**

Clinical and follow-up data were available in all patients and attempts were made to correlate nm23 expression with prognosis.

Statistical evaluation was performed by BMDP package program. The proportion surviving was estimated by Kaplan–Meier method and compared by Mantel–Cox test.

**Results**

**nm23 expression**

The level of nm23 expression was expressed as a percentage of the GAPDH mRNA level.

The mean level of nm23 expression in eight normal tissues samples, i.e. liver, breast, prostate, lymph node, spleen and ovary (65%) was approximately similar to the mean nm23 level in three benign naevi.

In the group with MM, expression was highly heterogeneous, ranging from 7% to 240% (Figure 1; Tables I and II).

**Clinical correlations**

A summary of statistical data is given in Table III.

Mean overall survival following metastasis resection was 21.6 months among the whole population of 30 patients. Within this population, patients with nm23 RNA content above the mean level of nm expression (46.9%) tended to do better than others: \( P = 0.08 \) (Figure 2 and Table II). Furthermore, among the 20 patients presenting with only regional lymph nodes (stage II) at the time of Northern blot analysis, there was a significant correlation between nm23 RNA level in the metastatic lymph node and the overall survival taking the node resection as a starting point. Indeed, stage II patients displaying nm23 expression above the mean level had a longer survival than others: \( P = 0.035 \) (Figure 3 and Table II).

Unlike the overall survival, the disease-free interval (from resection of the analysed metastasis to the occurrence of relapse) was not significantly different among stage II patients with nm23 expression above or below the mean level: \( P = 0.48 \) (Figure 4).

When the time of primary tumour resection was chosen as a starting point, a significant positive relation was observed between the time interval until the occurrence of the first metastasis and the nm23 level in this metastasis. Indeed, among the subgroup of patients who had presented initially as stage I (isolated cutaneous tumour) and evaluated for nm23 level in the first known metastasis (n = 15), the disease progression was slower in patients with nm23 above the median level (28%): \( P = 0.04 \) (Figure 5 and Table II). The median nm23 level was chosen as reference in this subgroup because all patients were above the mean level.

In addition, it must be noted that, at the time of lymph node metastasis resection, patients presenting with more disseminated disease (lymph node metastasis associated with involvement of other organs including skin) expressed lower nm23 levels (mean 31%) than patients harbouring a single lymph node metastasis (mean 51%), but the difference was not significant.

There was no significant correlation between nm23 expression and histological typing of primary cutaneous MM (Table I).

**Table 1** Correlations between nm23 expression in metastasis and histopathological characteristics of primary melanoma

| Cases | nm23 expression* | Histological type | Clark | Breslow (MM) |
|-------|-----------------|------------------|-------|-------------|
| 1     | 7               | NM               | IV    | 4           |
| 2     | 10              | NM               | IV    | 1.5         |
| 3     | 11              | SSM              | IV    | 2           |
| 4     | 14              | ALM              | III   | 1.5         |
| 5     | 14              | ALM              | III   | 1.4         |
| 6     | 16              | SSM              | IV    | 2.7         |
| 7     | 17              | ALM              | Y     | 5           |
| 8     | 20              | SSM              | II    | 0.6         |
| 9     | 22              | SSM              | II    | 0.8         |
| 10    | 22              | SSM              | III   | 1.4         |
| 11    | 22              | SSM              | IV    | 2.5         |
| 12    | 25              | SSM              | III   | 1.95        |
| 13    | 25              | NM               | IV    | 2.0         |
| 14    | 26              | NM               | II    | 0.9         |
| 15    | 28              | ALM              | IV    | 3.3         |
| 16    | 28              | NM               | III   | 2.4         |
| 17    | 29              | ALM              | IV    | 3.6         |
| 18    | 31              | SSM              | III   | 1.4         |
| 19    | 35              | SSM              | IV    | 1.4         |
| 20    | 41              | SSM              | III   | 1.4         |
| 21    | 46              | SSM              | IV    | 1.6         |
| 22    | 47              | ALM              | III   | 1.5         |
| 23    | 49              | SSM              | III   | 1.1         |
| 24    | 52              | SSM              | IV    | 2.5         |
| 25    | 63              | NM               | III   | 1.4         |
| 26    | 78              | NM               | IV    | 6           |
| 27    | 81              | SSM              | IV    | 5.8         |
| 28    | 88              | Unclassified     | 1     | 14          |
| 29    | 218             | Primary tumour unknown | 1       | 14          |
| 30    | 240             | NM               | III   | 1.4         |

*Analysed on early or late metastasis.

**Figure 1** Northern blot analysis showing the nm23 mRNA level in normal tissues, benign naevi and metastases of melanoma. Total RNA was hybridised to the 900 bp BamHI fragment of nm23-H1 cDNA (top) and as a control to a GAPDH probe (bottom). Lanes A–C, normal tissues from liver, breast and prostate; lanes D–F, benign naevi; lanes G–X, metastases of melanoma.
Table II Correlations between nm23 expression, overall survival from the time of nm23 analysis, disease-free interval from the time of primary tumor resection and clinical staging

| Cases | nm23 expression<sup>a</sup> | Overall survival (months)<sup>b</sup> | Interval from primary tumour<sup>c</sup> | Clinical staging<sup>d</sup> |
|-------|-----------------|-----------------|-----------------|-----------------|
| 1     | 7 (<m)          | 16              | II              |                |
| 2     | 10 (<m)         | 7               | II              |                |
| 3     | 11 (<m)         | 7               | II              |                |
| 4     | 14 (<m)         | 4               | 16              | III             |
| 5     | 14 (<m)         | 8               | II              |                |
| 6     | 16 (<m)         | 6               | III             |                |
| 7     | 17 (<m)         | 2               | II              |                |
| 8     | 20 (<m)         | 9               | III             |                |
| 9     | 22 (<m)         | 11              | 46              | II              |
| 10    | 22 (<m)         | 29              | II              |                |
| 11    | 22 (<m)         | 17              | 11              | III             |
| 12    | 25 (<m)         | 11              | 20              | II              |
| 13    | 25 (<m)         | 9               | III             |                |
| 14    | 26 (<m)         | 3               | 21              | III             |
| 15    | 28 (<m)         | 2               | II              |                |
| 16    | 28 (<m)         | 20              | III             |                |
| 17    | 29 (<m)         | 20              | 29              | II              |
| 18    | 31 (<m)         | 4               | 35              | II              |
| 19    | 35 (<m)         | 4               | 26              | II              |
| 20    | 41 (<m)         | 10              | 34              | II              |
| 21    | 46 (<m)         | 5               | 60              | II              |
| 22    | 47 (>m)         | 15              | 32              | II              |
| 23    | 49 (>m)         | 9               | 54              | III             |
| 24    | 52 (>m)         | 5               | III             |                |
| 25    | 63 (>m)         | 12              | 1               | II              |
| 26    | 78 (>m)         | 14              | III             |                |
| 27    | 81 (>m)         | 8               | II              |                |
| 28    | 88 (>m)         | 33              | II              |                |
| 29    | 218 (>m)        | 10              | II              |                |
| 30    | 240 (>m)        | 22              | 11              | II              |

<sup>a</sup> Analysed on early or late metastasis. (<m>) and (>m) refer to the mean level of nm23 expression (46.9%), calculated in the whole population of 30 samples. <sup>b</sup> From the time of metastasis resection, i.e. from nm23 Northern blot analysis. <sup>c</sup> Disease-free interval from resection of the primary cutaneous MM until occurrence of the first metastasis (restricted to 15 patients who had presented initially without metastasis and for whom nm23 analysis could be performed on the first metastasis). <sup>d</sup> Stage II, regional lymph node metastasis; stage III; visceral or disseminated metastases.

Table III Summary of statistical correlations between nm23 expression and patients' outcome

| Correlation between NM23 expression and | P-value<sup>1</sup> (Mantel–Cox) |
|----------------------------------------|----------------------------------|
| Overall survival from metastasis resection among all patients | 0.08 |
| Overall survival from metastasis resection among stage II patients | 0.035 |
| Disease-free survival from metastasis resection among stage II patients | 0.48 |
| Time interval from primary MM resection to first metastasis among stage I patients | 0.04 |

Discussion

Recent evidence indicates that the human nm23-H1 gene is located in 17q21.3, a chromosomal region known to contain the locus for early-onset familial breast–ovarian cancer and other genes involved in tumorigenesis (Steeg et al., 1988; Leone et al., 1991). This gene encodes one subunit of the enzyme NDP kinase (Gilles et al., 1991) and is structurally related to the human nm23-H2 gene encoding a second subunit of NDP kinase and co-localising with nm23-H1 in this region (Stahl et al., 1991). nm23 genes have also substantial homology with the predicted product of the Drosophila melanogaster developmental gene for abnormal wing discs (awd), which shows NDP kinase activity (Biggs et al., 1990).

It has been postulated that NDP kinase may participate in signal transduction through G-proteins (Stryer, 1986).

Although demonstration has been provided that the nm23 gene may act as a metastasis-suppressor gene in at least some experimental models (Henderson, 1993), the role of nm23 is still unclear in human cancer. Attempts to use tumour levels of nm23 expression as a predictive marker have given rise to contradictory findings.
In some breast tumours, evidence suggesting that low nm23 mRNA levels may indicate a poor prognosis could be demonstrated, based on the fact that patients whose tumours showed reduced nm23-H1 expression had a higher rate of lymph node metastasis and reduced survival (Bivlaacqua et al., 1989; Hennessy et al., 1991; Barnes et al., 1991). In colorectal carcinoma however, nm23 expression correlated only with the occurrence of liver metastasis but not with lymph node involvement (Haut et al., 1991; Yamagushi et al., 1993). In addition, human colon carcinomas were found to exhibit enhanced nm23 mRNA expression compared with normal mucosa (Yamagushi et al., 1993). Moreover, increased nm23 protein levels were observed, surprisingly, in advanced-stage neuroblastoma (Hailat et al., 1991).

A recent report has suggested that expression of the nm23 gene may be related to rapid progression in patients with MM. Florenes et al. (1992) observed that the nm23 mRNA level tended to be higher in secondary tumours occurring after prolonged relapse-free interval from primary diagnosis. Nonetheless, this study was only retrospective and did not attempt to show the usefulness of nm23 expression as a predictive parameter of prognosis.

The prognosis of patients with advanced MM actually remains poorly defined, since substantial variability in survival can be observed. In patients with regional nodal disease (stage II), the likelihood of systemic recurrence has been only correlated with the size and number of involved nodes, capsular effraction and more recently with some biological parameters (Sirot et al., 1993).

In the present report, we have tried to investigate the significance of nm23 expression as a prognostic marker for MM patients who have developed metastasis (stage II or III). We have therefore focused our study on the link between this expression and the time from biopsy of metastasis to the death of the patient (overall survival).

Our results proved to be of particular interest with regard to patients presenting with regional node invasion (stage II) at the time of Northern blot analysis. Among this subgroup, overall survival following metastasis resection was indeed significantly longer for patients with nm23 expression in metastasis above the mean level. These data are not only in accordance with a putative relationship between nm23 transcriptional level and progression of the disease, as suggested by Florenes et al. (1992), but they also provide the additional interest to be potentially helpful for the therapeutic strategy.

From a theoretical standpoint, some of our findings also seem noteworthy, although devoid of practical value. The fact that nm23 levels in the first known metastasis were related to the interval of time from primary MM diagnosis further supports the hypothesis that the nm23 gene may regulate at least some steps of the metastatic process in human MM.

Nonetheless, the mechanism by which the nm23 gene may be implicated in tumour progression still remains far from clear since, in contrast to what should have been expected, some of our MM metastasis samples exhibited higher level of nm23 expression than benign naevi and normal tissues. Similar findings were reported by Florenes et al. (1992). In this context, it must also be pointed out that nm23 expression in colon cancer can be higher than in normal surrounding mucosa (Yamagushi et al., 1993). An explanation for the low amounts of nm23 product which can be observed in normal or benign neoplastic tissue may be that the nm23 gene may play different roles in differentiated the malignant cells. With regard to the unexpectedly high nm23 RNA level in some aggressive tumours, it may also be suggested that nm23 molecular alterations, other than reduced expression, may result in aggressive tumoral behaviour. This hypothesis appears relevant in at least some cases of aggressive neuroblastoma harbouring nm23 genomic amplification and mutation (Hailat et al., 1991).

In conclusion, the present study suggests the prognostic value of nm23 expression in the practical management and therapeutic strategy of MM patients and should now be confirmed by larger series and clinical trials.

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