Downregulation of miR-125b in metastatic cutaneous malignant melanoma

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This study aimed to identify microRNA species involved in the earliest metastatic event in cutaneous malignant melanoma (MM). Samples from 28 patients with MM [stage T2 (tumor), M0 (distant metastasis)] were grouped by the presence of micrometastasis in the sentinel lymph nodes (N0/N1). Melanoma cells were harvested from primary, cutaneous MM tumors by laser-capture microdissection, and microRNA expression profiles were obtained by the microarray technique. Results were validated by quantitative reverse transcription PCR. We found that miR-125b was downregulated in the primary cutaneous melanomas that produced early metastases (T2, N1, M0) compared with the sentinel lymph node-negative (T2, N0, M0) melanomas. MiR-125b has earlier been found to be downregulated in other tumor types and in atypical naevi compared with the common acquired naevi. In conclusion, miR-125b may be involved in an early progression of cutaneous MM. 


Keywords: laser-capture microdissection, microRNA, miR-125b, melanoma

Introduction
Mortality from cutaneous malignant melanoma (MM) is in the range of 2.5/100 000 per year and this tumor ranks as one of the most important causes of death in western countries [1]. Tumor thickness and the presence of micrometastasis in the sentinel lymph nodes are the two major predictors of systemic dissemination and survival. Most patients with negative sentinel node status will be cured by radical surgical excision of the primary tumor, whereas 15% of the patients with the presence of lymph node micrometastasis will experience the recurrence of the disease (skin or distance metastases) [2]. Although it is not possible to distinguish between metastasizing and non-metastasizing melanoma on the basis of histology of the primary tumor, it has been argued that markers of lymphangiogenesis and melanocyte differentiation correlate with tumor aggressiveness in certain groups of melanoma patients [3].

MicroRNAs (miRNAs) comprise a newly discovered class of small size (18–25 nucleotides), noncoding RNA molecules [4], which regulate approximately 30% of human genes [5]. MiRNAs regulate gene expression by binding to imperfect complementary sites in the 3' untranslated region of their target messenger RNA transcript [6]. MiRNAs are involved in the regulation of multiple biological cellular processes, such as differentiation, proliferation, development, apoptosis, and cell-cycle [7]. Recent evidence has shown that aberrant expression or mutations of miRNA correlate with several human cancers and indicates that miRNAs can function as oncogenes or tumor suppressors [8–10].

Introduction of high-throughput technologies, such as microarray and deep sequencing technologies has revealed changes in miRNA expression patterns in cancer tissues. Different tumors show unique miRNA expression profiles, which is consistent with the tissue-specific functions of miRNAs [11]. Thus, miRNAs seem to be promising candidates for cancer biomarkers and therapeutic targets [12].

To identify potential miRNAs involved in progression of cutaneous MM, we analyzed miRNA expression levels in 28 formalin-fixed, paraffin-embedded samples of human primary cutaneous MMs (T2, N0/1, M0) (staging according to the American Joint Commission on Cancer: T2: Breslow thickness 1–2 mm, N0: no spread to lymph nodes, N1: spread to lymph node, M0: no distant metastasis). Each sample was microdissected to purify the melanoma cell population. By comparing the tumors, which metastasized to the lymph nodes (N1) with those who did not (N0), we identified nine differentially expressed miRNAs, including miR-125b. Validation by quantitative real-time PCR (qRT-PCR) yielded statistical significance for miR-125b indicating a potential role in early melanoma metastasis.

Methods
Patients and specimens
Formalin-fixed paraffin-embedded samples of MM were identified from the Danish Melanoma Group database and the pathology databases in our institutions. We
identified samples from 28 patients (15 women and 13 men, age range 24–82 years, mean 52.5 years) diagnosed between 2003 and 2008 with cutaneous MM in stage II: T2, N0/1, M0 (Table 1). All samples were reviewed by an expert pathologist (NH). The study was approved by the Danish National Committee on Biomedical Research Ethics.

**Laser-capture microdissection**

The paraffin-embedded melanoma samples were sectioned at 10 μm and mounted on MMI RNase free membrane slides (MMI Molecular Machines & Industries AG, Glattbrugg, Switzerland). Slides were deparaffinized with xylene, fixed in ethanol, stained with Mayer’s hematoxylin and eosin according to the MMI H and E Staining Kit for laser-capture microdissection (LCM) protocol. LCM was performed using Olympus microscope Laser-capture system following manufacturer’s instruction (Olympus, Tokyo, Japan). The described staining technique allowed for histological identification of melanoma cells for microdissection. We captured 20–50 000 melanoma cells from each specimen.

**RNA isolation**

We have earlier described the method of RNA isolation from the paraffin-embedded skin samples for miRNA microarray profiling [13]. Total RNA was extracted from each microdissected sample using a combination of Ambion’s RNAqueous-Micro Kit and RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems/Ambion, Austin, Texas, USA). Briefly, protein–RNA cross links were removed by protease/digestion buffer treatment and RNA was purified on a column and eluted in 20 μl of nuclease-free water. Total RNA yield and quality were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) with an inclusion criterion of A260/280 ≥ 1.6.

**microRNA microarray**

About 200 ng of total RNA was labeled with miRCURY LNA microRNA Array Power Labeling Kit (Exiqon A/S, Vedbaek, Denmark) using fluorescent Hy3(sample)/Hy5(reference) dyes, according to the manufacturer’s instructions. Labeled samples were then hybridized overnight to preprinted miRCURY LNA microRNA Array, V10.0 (Exiqon), containing probes for human miRNAs, cataloged in the miRBase Sequence Database (Release 10.0, http://microrna.sanger.ac.uk/), and a number of proprietary human miRPlus sequences not yet annotated in miRBase.

**Image analysis**

After hybridization, the arrays were scanned with an Agilent DNA Microarray Scanner (Agilent Technologies, Inc., Santa Clara, California, USA) and resulting images were analyzed using GenePix Pro 6.1 (Molecular Devices, Sunnyvale, California, USA). Each spot intensity signal was measured as median of foreground intensities, using the morphological open/close method. To obtain an estimate of the true spot intensity, we used the subtract method for background correction [14]. Normalization was performed during statistical analysis.

**Quantitative real-time-PCR**

cDNA was prepared from total RNA samples using TaqMan MicroRNA Reverse Transcription Kit on the Applied Biosystems 7900 HT Fast Real-Time PCR System according to manufacturer’s instructions (Applied Biosystems, Foster City, California, USA). Predesigned TaqMan MicroRNA Assays for hsa-miR-125b and control hsa-miR-191 were purchased from Applied Biosystems (Applied Biosystems). qRT-PCR was performed using TaqMan Universal PCR Master Mix, according to the manufacturer’s protocol.

**Statistical analysis**

Statistical analysis of microarray data was performed using the R software version 2.9.2. Background corrected intensity data were normalized using Loess normalization [15] and between arrays using quantile normalization [16]. MiRNAs that were differentially expressed between the melanoma groups [negative and positive sentinel node biopsy (SNB)] were identified using a linear Bayesian model. Derived P values were adjusted using Benjamini–Hochberg false discovery rate (FDR) correction with a cut-off at 0.1 [17]. qRT-PCR results were imported into Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA). Statistical analysis was performed on Microsoft Excel and R (The R Foundation for Statistical Computing, Vienna, Austria).

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**Table 1 Sample data**

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Melanoma type</th>
<th>Clark level</th>
<th>Breslow (mm)</th>
<th>SNB status</th>
</tr>
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<tbody>
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<td>Positive</td>
</tr>
<tr>
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<td>SSM</td>
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<td>1.50</td>
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</tbody>
</table>

All samples are primary cutaneous malignant melanoma (T2, N0/1, M0). SNB, sentinel node status; SSM, superficial spreading melanoma.
Washington, USA). Raw C\textsubscript{T} values converted to 2\textminus\Delta C\textsubscript{T} values [18] using miR-191 as the internal control [19]. Student’s \textit{t}-test was performed using SAS/STAT Software (SAS Institute Inc., Cary, North Carolina, USA) and a \textit{P} value less than 0.05 was used for significance.

**Results**

For microarray analysis, we purified MM cells by LCM from 28 patients with T2, M0 cutaneous MM (Fig. 1, Table 1). Material containing typically 20–50 000 melanoma cells yielded 700–2400 ng of total RNA. For our reference samples, the total yield of RNA was in the range of 1500–10 000 ng. The optical densities (A260/A280) for the LCM samples were in the range of 1.62–2.01.

In our melanoma samples, a total of 401 miRNAs were expressed. When comparing miRNA expression profiles of the SNB-negative (\(n = 14\)) and SNB-positive groups (\(n = 14\)), nine miRNAs were significantly differentially expressed (FDR) less than 0.1 (Table 2). Eight of them (miR-92a, miR92b, miR-125b, miR-125a-5p, miR-196a\textsuperscript{∗}, miR-211, miR-204, and miR-625\textsuperscript{∗}) were downregulated in the SNB-positive group compared with the SNB-negative group while miR-548c-3p was upregulated (Fig. 2). As material was scarce and ethical reasons hindered more use of our melanoma samples, it was only possible to validate the top five significantly differentially expressed miRNAs by qRT-PCR. Validation of miR-196a\textsuperscript{∗} failed for technical reasons. For the remaining four miRNAs, deregulation was comparable with our microarray data; however, only miR-125b reached a significant level. Figure 3 shows the validation results for miR-125b. As seen, miR-125b is significantly downregulated in the SNB-positive group compared with the SNB-negative group [fold change (FC) 3.5, \(P < 0.05\)]. This result is in good correlation with the microarray data, where miR-125b was downregulated in the SNB-positive group by a FC of 1.58 (FDR < 0.1) (Fig. 4). Using Targetscan (http://targetscan.org) and DIANA-microT V 4.0 (http://diana.cslab.ece.ntua.gr), the online resources for in-silico miRNA target prediction and pathway analysis, we looked up whether miR-125b targets messenger RNAs relevant for MM. Among hundreds of theoretical targets for miR-125b, we found ErbB, Notch, p53, and mitogen-activated protein kinase-signaling pathways, including AKT3, MCL1, BCL2, PPP2CA, Notch 2, and members of the ErbB family (Table 3) [20–26].

**Discussion**

It has recently become evident that aberrant expression of certain miRNAs contributes to the initiation and progression of cancer. Many studies have sought to identify unique miRNA signatures with diagnostic or prognostic value in many diseases, including MM. Segura \textit{et al.} [27]...
showed that in late MM (stage III/IV) six miRNAs (miR-145, miR-150, miR-155, miR-432-3p, miR-455-3p, and miR-497) predicted the postrecurrence survival. Satzger et al. [28] analyzed the expression of 16 selected miRNAs in melanocytes and melanoma cell lines and found miR-15b to be associated with poor prognosis. Addressing the possible role of miRNA in melanoma progression, Mueller et al. [29] analyzed miRNome changes in melanoma cell lines derived from primary melanomas and melanoma metastases. The authors were able to identify sets of miRNAs potentially involved in malignant transformation, tumor progression, and metastasis. By comparing miRNA expression levels in primary melanoma cell lines with those in melanoma cell lines derived from metastatic melanomas, they found 13 aberrantly expressed (FC > 3) miRNAs in metastatic melanoma cell lines. Chen et al. [30] discovered 18 deregulated miRNA in metastatic melanoma samples when compared with benign nevi, including miR-125b, miR-204, and miR-211.

In this study, we compared miRNA expression profiles in two groups of patients with primary cutaneous MM (T2, M0): the SNB-positive (N1) and SNB-negative (N0) tumors to identify the differentially expressed miRNAs that may be involved in the early metastatic behavior of the primary tumor. To our knowledge, this is the first report of genome-wide miRNA profiling in LCM samples of human primary cutaneous MM. As miRNA are highly cell and tissue-specific, we used microdissection technique to avoid contamination with the RNA originating from stromal cells. Despite the limited amount of the starting material, we succeeded in extracting enough total RNA of good quality for microarray analysis. The expression patterns in the SNB-positive and SNB-negative groups were very similar (correlation coefficient > 0.9), indicative of a good reproducibility of the method. Eight miRNAs (miR-125b, miR92b, miR-125b, miR-125a-5p, miR-196a, miR-211, miR-204, and miR-625) were significantly downregulated in SNB-positive melanomas compared with the SNB-negative group, whereas miR-548c-3p was upregulated. Our dataset contained three miRNAs (miR-125b, miR-211, and miR-204), which earlier have been reported deregulated in melanoma samples or cell lines [30]. In addition, miR-92a has been recognized as a controller of angiogenesis and inhibition of miR-92a enhanced tumor vessel growth in a mouse model [31].

As described above miR-125b was the only miRNA species, the downregulation of which in the SNB-positive group could be validated by qRT-PCR. We consider miR-125b to be involved in the early progression of melanoma, especially in view of our recent data showing

### Table 3 Predicted target genes in pathways relevant for development and progression of cutaneous malignant melanoma

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene name</th>
</tr>
</thead>
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<td>ErbB signaling pathway</td>
<td>NRG3, EIF4EBP1, ABL2, MAP2K7,</td>
</tr>
<tr>
<td></td>
<td>AKT3, ERBB members</td>
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<tr>
<td>Notch signaling pathway</td>
<td>DLX3, DTX4, LFNG, NOTCH2</td>
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<td>MAPK signaling pathway</td>
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<tr>
<td></td>
<td>CDC25B, MAP2K7, DUSP3, DUSP6,</td>
</tr>
<tr>
<td></td>
<td>AKT12, AKT3</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>BCL2, AKT3</td>
</tr>
<tr>
<td>Melanoma</td>
<td>AKT3</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>DDB2</td>
</tr>
<tr>
<td>Other</td>
<td>PPP2CA, MCL1</td>
</tr>
</tbody>
</table>

MAPK, mitogen-activated protein kinase.
that expression of miR-125b is downregulated in atypical nevi compared with the sporadic acquired nevi (Holst et al. in preparation). In addition, our researchers independently reported that miR-125b is deregulated in MM. Schultz et al. [32] found that miR-125b is 10-fold downregulated in the primary melanomas compared with benign melanocytic nevi. A 5-fold downregulation of miR-125b in the metastatic melanoma cell lines comparing with primary melanomas was registered by Philippidou et al. [33]. Another study by Chen et al. [30] profiled 470 miRNA in 16 benign nevi and metastatic melanomas. Among 18 differentially expressed miRNA, miR-125b was expressed 2-fold lower in the metastatic samples. Interestingly, miR-125b has been found deregulated in several other tumor types, such as hepatocellular carcinoma, anaplastic thyroid carcinoma, breast cancer, ovarian carcinoma, bladder, and oral cancer [34–40]. Potential involvement of miR-125b in MM progression is further reinforced by in-silico analysis of its gene targets. Table 3 shows some of the predicted targets of miR-125b and their relation to the pathways known to be involved in melanoma. To our knowledge, none of these has earlier been experimentally tested in miR-125b melanoma studies. However, overexpression of miR-125b in an ErbB2 positive breast cancer cell line impaired cell growth and mobility [41]. This is interesting, as ErbB2 and ErbB3 have been suggested to be of importance in the pathogenesis of melanoma [25,26]. AKT3 is also of interest, as increased AKT activity is found in 50% of melanomas. AKT3 expression correlates with melanoma progression, whereas depletion of AKT3 induces apoptosis in melanoma cells and reduces the growth of xenografts [42]. Functional studies are needed to explore the effect of miR-125b with regard to these melanoma-related target genes.

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Conflicts of interest: none declared.

References


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