

MicroRNA Expression Profile in Conjunctival Melanoma

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PURPOSE. Conjunctival melanoma (CM) is a rare disease associated with considerable mortality. As opposed to cutaneous melanoma, the epigenetic mechanisms involved in the development of CM and other mucosal melanomas (MMs) are unclear. The purpose of this study was to identify tumor-specific and prognostic microRNA (miRNA) in CM and to compare the miRNA profile with that of MM.

METHODS. Using microarray analysis (Affymetrix) we determined the miRNA expression profile in 40 CMs compared with 7 normal conjunctival samples. Changes in miRNA expression were associated with T stage, local recurrence, metastasis, and mortality. Furthermore, the expression of six fresh frozen tissue samples of CM was compared with that of four laryngeal and sinonasal MM.

RESULTS. Our analysis revealed 24 upregulated and 1 downregulated miRNA in CM; several of these miRNAs have key functions in the pathogenesis and progression of cutaneous melanoma. Additionally, we identified seven upregulated miRNAs specific for stage-T1 and stage-T2 CM, whose expression was associated with increased tumor thickness ($P = 0.007$), and two upregulated miRNAs (miR-3687 and miR-3916) associated with an increased risk of local recurrence. No stage T3-specific miRNAs were identified.

CONCLUSIONS. We identified differentially expressed and potentially prognostic miRNAs in CM. Furthermore, the miRNA expression pattern of CM resembled that in MM. The identification of these differentially expressed miRNAs provides an entry point for future functional studies of miRNAs as prognostic or therapeutic targets in CM and highlights the resemblance between CM, MM, and cutaneous melanoma.

Keywords: conjunctiva, melanoma, microRNA, gene expression

Conjunctival melanomas (CMs) account for approximately 5% of ocular melanomas and they have an associated mortality of up to 30%.^{1,2} Recently, oncogenic mutations in *BRAF*, *NRAS*, and *KIT* have been identified in CM, emphasizing their close genetic resemblance to cutaneous and mucosal melanomas (MMs), and their difference from uveal melanomas (UMs).^{2–5} Owing to the high mortality rate associated with CM and MM,^{2,6} there is a need to further identify molecular pathways that drive development and progression. MicroRNAs (miRNAs) are small noncoding RNA molecules that epigenetically regulate gene expression at the posttranscriptional level by either degradation of or translational blockage of target messenger RNAs.⁷ Deregulation of miRNAs with oncogenic and tumor-suppressive functions have attracted much attention owing to their high prevalence.⁸ These small miRNA molecules could be used as prognostic or therapeutic targets. A microarray-based miRNA expression profiling platform was therefore used to compare the expression of miRNAs in archived (formalin-fixed, paraffin-embedded [FFPE]) CM and normal conjunctival samples. The miRNA expression pattern in CM was tested for associations with TNM stage,⁹ local recurrence,

metastasis, and mortality in order to identify prognostic miRNAs. To determine similarities in miRNA expression between CM and different subtypes of MM, fresh frozen tissue samples from CM, sinonasal MM, and laryngeal MM were analyzed and the miRNA expression results were compared.

MATERIALS AND METHODS

Patients and Tissue Samples

Patients diagnosed with CM were identified by searching the archives of the Eye Pathology Institute and the Danish Registry of Pathology. Archived (2000–2012) and fresh frozen (2000–2014) tissue samples were collected. Archived tissue samples from normal conjunctiva were collected from the Eye Pathology Institute (1999–2000) from patients with no history of a pigmented lesion. Fresh frozen tissue samples from sinonasal and laryngeal MM patients were identified and obtained from the Danish Registry of Pathology and the Danish Cancer Biobank (2000–2014).



Information on patient and tumor characteristics, clinical presentation, local recurrence, regional and distant metastases, and cause of death was obtained from clinical patient records, from pathology reports, and by searching the Danish Registry of Pathology and the Danish Register of Causes of Death, as previously described.^{2,10} The proliferative index (mitotic rate) was evaluated in slides stained with anti-Ki-67 (Dako Denmark, Glostrup, Denmark) and the percentage of Ki-67-positive cells was assessed. All of the CMs were divided into two groups ($\leq 15\%$ and $> 15\%$). TNM staging was based on clinical information available and was performed according to the American Joint Commission on Cancer TNM classification, seventh edition.⁹

We obtained sufficient archived FFPE tumor material from 40 of 55 CM samples and from 7 normal conjunctival samples. Fresh frozen tumor samples were obtained from six CMs, three sinonasal melanomas, and one laryngeal melanoma. Archived CM samples were evaluated with hematoxylin-eosin- and Melan-A-stained slides to establish the amount of tumor tissue. Then 8 to 10 sections, each of 20- μ m thickness, were cut. When the amount of tumor tissue in the sample was evaluated to be $< 80\%$ to 90% , the sections were transferred to glass slides and tumor tissue was macrodissected by hand with a scalpel. The study adhered to the tenets of the Declaration of Helsinki. Ethical Committee approval was obtained and the study was approved by the Danish Data Protection Agency.

RNA Isolation

For automated purification of total RNA from FFPE tissue, we used the QIASymphony RNA Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). For purification of total RNA (including small RNAs) from fresh frozen tumor samples, the AllPrep DNA/RNA Micro Kit was used according to the manufacturer's instructions (also Qiagen). The degree of RNA degradation was evaluated before microarray analysis by using the Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Microarray Profiling Analysis

One hundred thirty micrograms of total RNA was prepared from each sample and hybridized on a GeneChip 4.0 Array (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. Briefly, total RNA was labeled with FlashTag Biotin HSR by Poly(A) tailing and subsequent FlashTag Biotin HSR ligation, and incubated at 99°C for 5 minutes and 45°C for 5 minutes with Hybridization Master Mix. Arrays were hybridized at 48°C and 60 rpm for 16 to 18 hours in an Affymetrix 640 Hybridization oven, and washed and stained with phycoerythrin-conjugated streptavidin in an Affymetrix 450 Fluidics Station. The arrays were scanned in an Affymetrix GeneChip Scanner 3000 to generate fluorescent images, as described in the Affymetrix Gene Chip protocol. Cell intensity files were generated with GeneChip Command Console software (AGCC) (Affymetrix). Cell intensity files were RMA-normalized with the Affymetrix Expression Console, and 2578 probe sets representing human mature miRNAs were selected for further analysis. The expression matrix was imported into the R environment¹¹ and differential expression between samples was defined with eBayes modeling, as implemented in the R LIMMA package.¹² MicroRNAs were defined as showing differential expression if fold changes were above 2 and Benjamini-Hochberg-adjusted *P* values (false discovery rate) were below 0.1. Differentially expressed miRNAs were visualized by using hierarchical clustering based on average linkage and Euclidian distance, as implemented in Qlucore

Omics Explorer (www.qlucore.com; provided in the public domain by Qlucore, Lund, Sweden).

Analysis of Archived Tissue Samples

For the analysis of miRNA expression in CMs from archived tissue, three heavily pigmented CMs were excluded because miRNA signals were disturbed. This resulted in the inclusion of 37 CM samples for further analysis. For the identification of tumor-specific miRNAs, all pooled tumor samples were compared to normal conjunctiva. For the identification of prognostic miRNAs, tumors of TNM stages T1, T2, and T3 were compared to normal conjunctiva. In addition, the associations of each miRNA with local recurrence, distant metastasis, any metastasis (regional or distant), and melanoma-related mortality were tested by Cox regression analysis, as implemented in the R survival package. χ^2 and Kruskal-Wallis tests were used to compare patient characteristics and tumor features with clusters of low, intermediate, and high miRNA expression. Statistical calculations were performed by using SPSS (version 20; IBM, Armonk, NY, USA).

Analysis of Fresh Frozen Tumor Samples

Fresh frozen tissue samples were analyzed as an additional part of the study. Expression analysis was performed as described for archived tissue, and the expression of tumor-specific miRNAs was compared to CM, sinonasal MM, and laryngeal MM. In three CM patients, miRNA expression results were obtained from both fresh frozen and archived tissue samples. We were therefore able to measure the correlation coefficient (*r*) between miRNA expression from fresh frozen CM samples and archived CM samples by using Pearson product-moment correlation analysis.

RESULTS

Patient and Tumor Characteristics

MicroRNA expression results were obtained in 37 consecutive CM patients, 17 men and 20 women, with a median age of 72 years (range, 37–92 years). The median follow-up period was 3.7 years (range, 5 months–13 years). The tumors were most commonly epibulbar (27/37), and local invasion to eyelid skin occurred in four cases. The tumors were staged as T1 (27), T2 (6), and T3 (4). Median tumor thickness was 1.2 mm (range, 0.2–8.0 mm); the CM most commonly originated in a primary acquired melanosis with atypia (PAM+) (30/37), and a third of the CMs (12/37) were *BRAF* mutated (Table 1). There was local recurrence in 14/37 cases (38%), regional metastasis was recorded in three patients, and distant metastasis developed in five patients. At the end of follow-up, 4 patients had died from melanoma-related causes, 12 patients had died from other causes, 2 patients had died from unknown causes, and 19 patients were still alive.

Differentially Expressed miRNAs in Conjunctival Melanoma

We identified 25 miRNAs in CM that were differentially expressed relative to normal conjunctiva (24 upregulated and 1 downregulated) (Table 2). We performed a supervised hierarchical cluster analysis of the 25 differentially expressed miRNAs. Six of seven of the normal tissue samples clustered closely together, with low expression of 24 miRNAs and high expression of 1 miRNA. The tumor samples were clustered according to expression levels and showed a gradient of

TABLE 1. Demographic, Clinicopathologic, and Genetic Characteristics of 37 Consecutive Conjunctival Melanoma Patients Who Were Tested for Associations With Expression of Tumor-Specific miRNAs

| Characteristic | Total Patients, No. (%) | MicroRNA Expression Group,* No. (%) | | | P Value† |
|------------------------------------|-------------------------|-------------------------------------|---------------|---------------|----------|
| | | Low | Intermediate | High | |
| Sex | | | | | |
| Male | 17 (46) | 4 (36) | 5 (42) | 8 (57) | 0.55 |
| Female | 20 (54) | 7 (64) | 7 (58) | 6 (43) | |
| Age, mean (95% CI), y | 70 (64–75) | 71 (60–82) | 69 (58–79) | 70 (60–80) | 0.86 |
| Tumor location | | | | | |
| Epibulbar | 27 (73) | 7 (64) | 11 (92) | 9 (64) | 0.26 |
| Extrabulbar | 9 (24) | 3 (27) | 1 (8) | 5 (36) | |
| Caruncular | 1 (3) | 1 (9) | 0 | 0 | |
| Local invasion to eyelid skin | | | | | |
| Present | 4 (11) | 2 (18) | 1 (8) | 1 (7) | 0.64 |
| Absent | 33 (89) | 9 (82) | 11 (92) | 13 (93) | |
| TNM stage‡ | | | | | |
| T1 | 27 (73) | 7 (64) | 11 (92) | 9 (64) | 0.29 |
| T2 | 6 (16) | 2 (18) | 0 | 4 (29) | |
| T3 | 4 (11) | 2 (18) | 1 (8) | 1 (7) | |
| Tumor thickness, mean (95% CI), mm | 1.6 (1.2–2.2) | 1.0 (0.5–1.5) | 1.2 (0.6–1.7) | 2.7 (1.6–3.8) | 0.007 |
| Cell type | | | | | |
| Epithelioid | 16 (43) | 2 (18) | 8 (67) | 6 (43) | 0.16 |
| Mixed | 18 (49) | 7 (64) | 4 (33) | 7 (50) | |
| Spindle | 3 (8) | 2 (18) | 0 | 1 (7) | |
| Ki-67 proliferative index | | | | | |
| ≤15, low | 21 (62) | 8 (89) | 8 (67) | 5 (48) | 0.052 |
| >15, high | 13 (38) | 1 (11) | 4 (33) | 8 (62) | |
| Origin | | | | | |
| PAM+ | 30 (81) | 11 (100) | 10 (84) | 9 (64) | 0.26 |
| Nevus | 4 (11) | 0 | 1 (8) | 3 (22) | |
| De novo | 3 (8) | 0 | 1 (8) | 2 (14) | |
| <i>BRAF</i> | | | | | |
| Mutated | 12 (32) | 1 (9) | 5 (42) | 6 (43) | 0.14 |
| Wild-type | 25 (68) | 10 (91) | 7 (58) | 8 (57) | |

* Low, intermediate, or high expression of seven upregulated microRNAs identified in conjunctival melanoma.

† Fisher's exact test, χ^2 test, or Kruskal-Wallis test as appropriate.

‡ None of the patients had nodal or distant metastasis at the time of diagnosis.

increased expression from melanomas resembling normal tissue to a highly upregulated expression pattern of 24 miRNAs and downregulated expression of 1 miRNA.

Prognostic Significance of Differentially Expressed miRNAs

To determine the prognostic associations of differentially expressed miRNAs, stage-specific miRNAs were identified (Fig. 1). Seven miRNAs (miR-30d-5p, miR-138-5p, miR-146a-5p, miR-500a-5p, miR-501-3p, miR-501-5p, and miR-502-3p) were significantly and simultaneously upregulated in both stage-T1 and stage-T2 CM relative to normal conjunctiva (Table 2). A 2-way cluster analysis based on these seven differentially expressed miRNAs distinguished the samples in clusters of low expression ($n = 11$), intermediate expression ($n = 12$), and high expression ($n = 14$) (Fig. 1). Six of seven (85%) of the normal conjunctival samples clustered in the low expression group. The tumors showed a gradient increase from low expression to high expression. In particular, 14 CM samples showed a high expression pattern. These were significantly thicker (mean thickness

of 2.7 mm) than 12 tumors with intermediate expression and 11 tumors with low expression (mean tumor thickness of 1.0 and 1.2 mm, respectively) ($P = 0.007$, Kruskal-Wallis). Additionally, the CMs with a high expression pattern tended to have a higher Ki-67 proliferative index ($P = 0.052$). No other demographic features or tumor characteristics were significantly associated with the expression patterns observed. Expression of a single miRNA (miR-5096) was downregulated in T2 tumors relative to normal conjunctiva. No significant difference in miRNA expression was identified when we compared stage-T3 tumors ($n = 4$) with normal conjunctiva.

An upregulated expression of miR-3687 and miR-3916 was associated with a higher risk of local recurrence in CM (Table 3). None of the miRNAs were significantly associated with metastasis, or with melanoma-related mortality.

MicroRNA Expression in Conjunctival Melanomas and in Other Mucosal Melanomas

Fresh frozen tissue samples were obtained from six CMs, three sinonasal MMs, and one laryngeal MM. A highly

TABLE 2. MicroRNAs With Significantly Different Expression in CM and Normal Conjunctiva (Normal), miRNAs With Significantly Different Expression in CM Stages T1 and T2 Relative to Normal, and Associations With miRNA Expression Observed in Cutaneous Melanoma

| miRNA Conjunctival Melanoma | Fold Change | P Value* | Reference—miRNAs Associated With Cutaneous Melanoma |
|-----------------------------|-------------|----------------------|--|
| CM vs. normal | | | |
| Hsa-miR-3689 | 4.1 | 7.5×10^{-6} | |
| Hsa-miR-181b-5p | 13.3 | 1.4×10^{-5} | |
| Hsa-miR-132-3p | 4.9 | 2.1×10^{-5} | Pinto et al. ²⁷ |
| Hsa-miR-510-5p | 8.2 | 2.9×10^{-5} | |
| Hsa-miR-138-5p | 21.6 | 3.3×10^{-5} | Poliseno et al. ²⁸ |
| Hsa-miR-363-5p | 10.0 | 7.7×10^{-5} | Philippidou et al. ²⁶ |
| Hsa-miR-345-5p | 4.5 | 8.1×10^{-5} | |
| Hsa-miR-4689 | -2.1 | 0.0001 | |
| Hsa-miR-181a-2-3p | 6.2 | 0.0002 | |
| Hsa-miR-501-3p | 4.9 | 0.0002 | |
| Hsa-miR-146b-5p | 6.0 | 0.0002 | Chen et al. ²¹ ; Philippidou et al. ²⁶ ; Poliseno et al. ²⁸ ; Saleiban et al. ¹⁵ ; Sand et al. ³⁰ |
| Hsa-miR-509-3p | 272.4 | 0.0002 | Saleiban et al. ¹⁵ ; Streicher et al. ¹⁶ |
| Hsa-miR-146a-5p | 74.4 | 0.0002 | Forloni et al. ²³ ; Philippidou et al. ²⁶ ; Poliseno et al. ²⁸ ; Qi et al. ²⁹ ; Raimo et al. ³¹ |
| Hsa-miR-500a-5p | 5.8 | 0.0003 | |
| Hsa-miR-509-3-5p | 24.4 | 0.0003 | Saleiban et al. ¹⁵ |
| Hsa-miR-532-3p | 4.8 | 0.0004 | |
| Hsa-miR-181a-5p | 9.0 | 0.0004 | Poliseno et al. ²⁸ |
| Hsa-miR-500b-3p | 4.7 | 0.0005 | Saleiban et al. ¹⁵ |
| Hsa-miR-20b-5p | 7.0 | 0.0005 | Saleiban et al. ¹⁵ ; Xu et al. ¹⁸ |
| Hsa-miR-506-3p | 14.1 | 0.0006 | Streicher et al. ¹⁶ |
| Hsa-miR-128-3p | 5.0 | 0.0007 | |
| Hsa-miR-532-5p | 5.1 | 0.0007 | Kitago et al. ²⁵ ; Saleiban et al. ¹⁵ |
| Hsa-miR-502-3p | 5.6 | 0.0007 | |
| Hsa-miR-1260b | 4.2 | 0.001 | |
| Hsa-miR-501-5p | 5.4 | 0.001 | Saleiban et al. ¹⁵ |
| T1 and T2 vs. normal† | | | |
| Hsa-miR-138-5p | 9.0 | 3.5×10^{-5} | Poliseno et al. ²⁸ |
| Hsa-miR-501-3p | 3.5 | 4.4×10^{-5} | |
| Hsa-miR-146a-5p | 11.1 | 9.4×10^{-5} | Forloni et al. ²³ ; Philippidou et al. ²⁶ ; Poliseno et al. ²⁸ ; Qi et al. ²⁹ ; Raimo et al. ³¹ |
| Hsa-miR-30d-5p | 4.4 | 0.0002 | Fleming et al. ²² ; Gaziel-Sovran et al. ²⁴ ; Philippidou et al. ²⁶ ; Poliseno et al. ²⁸ |
| Hsa-miR-502-3p | 4.1 | 0.0003 | |
| Hsa-miR-500a-5p | 3.6 | 0.0003 | |
| Hsa-miR-501-5p | 3.9 | 0.0003 | Saleiban et al. ¹⁵ |

Positive fold changes represent upregulation and negative fold change represents downregulation of miRNA expression in tumors relative to normal tissue. MicroRNAs in bold have been reported previously in cutaneous melanoma.

* Significantly expressed miRNAs were identified after application of a false discovery rate less than 0.1 and a fold change of 2.0 or greater.

† T stages were designated according to the American Joint Commission on Cancer TNM classification (7th edition), and the reported P values and fold changes were from the comparison of tumor stage T2 with normal.

significant correlation in expression patterns from fresh frozen CM samples and archived CM samples from three patients was observed, with Pearson correlation coefficients (r) of 0.85 (95% confidence interval [CI]: 0.84–0.86, $P < 2.2 \times 10^{-16}$), 0.91 (95% CI: 0.90–0.91, $P < 2.2 \times 10^{-16}$), and 0.93 (95% CI: 0.92–0.93, $P < 2.2 \times 10^{-16}$). Most of the CMs were from female patients. The patient ages ranged from 34 to 92 years, and the tumors were most frequently located in the epibulbar region. Three of four sinonasal and laryngeal MM patients were males, and patient age ranged from 65 to 87 years (Table 4). No significant difference in expression was identified when comparing the expression of the 25 differentially expressed miRNAs (upregulated in CM relative to normal conjunctiva) with the miRNA expression results obtained from sinonasal and laryngeal MMs (Fig. 2).

DISCUSSION

Recently, the identification of *BRAF*, *NRAS*, and *KIT* mutations in CM has shown a striking difference from UMs and has highlighted the genetic resemblance with both cutaneous and mucosal melanoma.^{2–5,13,14} Identification of diagnostic, prognostic, or therapeutic miRNAs has the potential to improve the prognosis for both cutaneous melanoma patients and UM patients.^{15–18} However, to the best of our knowledge, no previous reports on miRNA expression in CM and head and neck MM have been published.

The CM patients included in the present study had a sex ratio of approximately 1:1, and apart from having a slightly higher age on average at diagnosis, the present data may be regarded as being representative of CM.^{2,19,20}

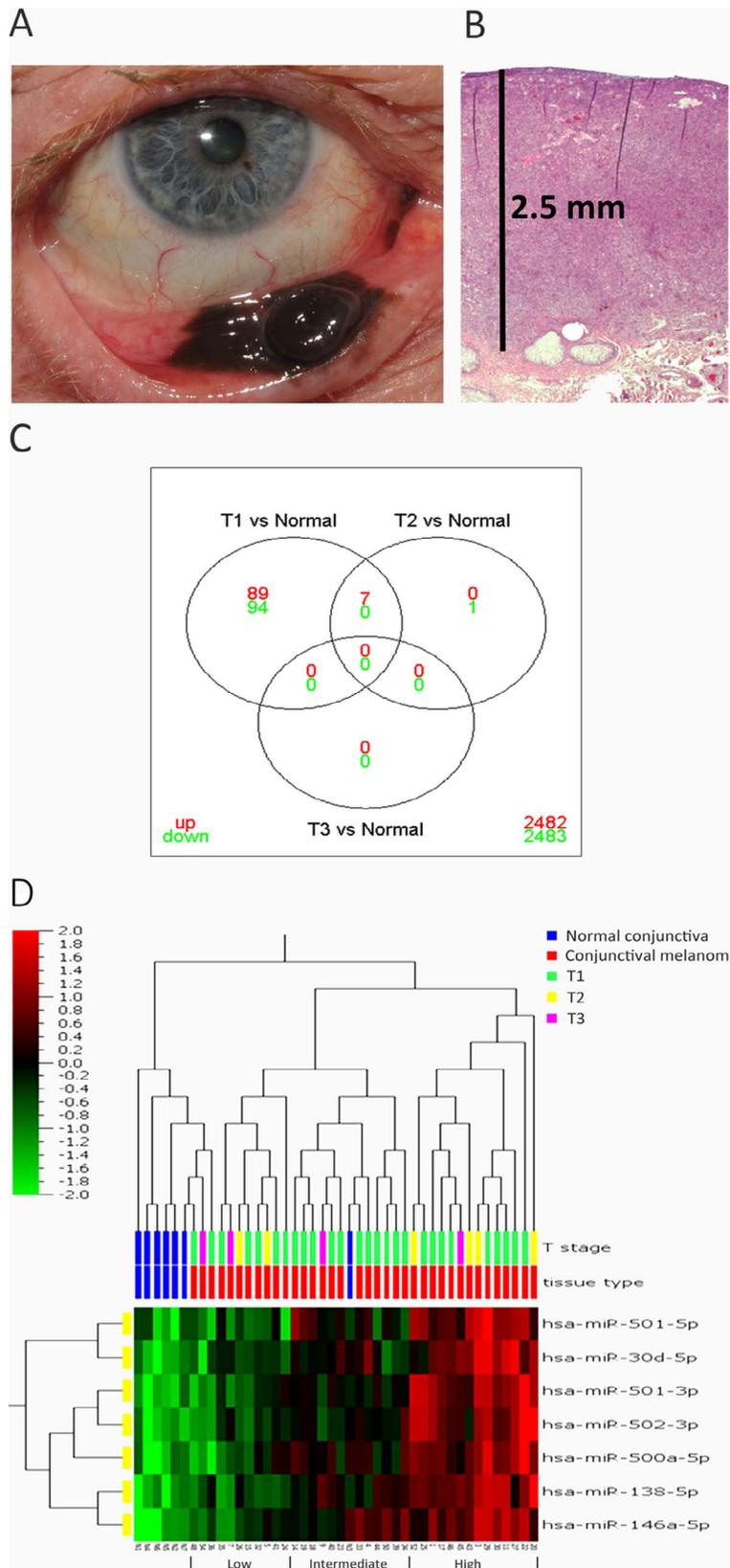


FIGURE 1. MicroRNAs with a significantly different degree of expression between CM stage T1, T2, and T3 and normal conjunctiva (false discovery rate < 0.1, fold change > 2). **(A)** Nodular melanoma of the palpebral conjunctiva (stage T2) with melanosis of the lid margin and plica semilunaris. **(B)** Conjunctival melanoma with a tumor thickness of 2.5 mm. Hematoxylin-eosin, original magnification $\times 2.5$. **(C)** Venn diagram showing seven upregulated miRNAs (miR-30d-5p, miR-138-5p, miR-146a-5p, miR-500a-5p, miR-501-3p, miR-501-5p, and miR-502-3p) overlapping between stage-T1 and stage-T2 CM, and a single downregulated miRNA (miR-5096) in stage-T2 CM. **(D)** Hierarchical cluster visualization of the seven significantly upregulated miRNAs divided the CM in groups of low, intermediate, and high expression.

TABLE 3. MicroRNAs Associated With an Increased Risk of Local Recurrence in Cox Regression Analysis

| MicroRNA | Cox Coefficient | Cox P Value* |
|--------------|-----------------|-----------------------|
| Hsa-miR-3687 | 2.14 | 5.20×10^{-5} |
| Hsa-miR-3916 | 4.46 | 3.96×10^{-6} |

* Significantly expressed miRNAs were identified after application of a false discovery rate less than 0.1 and a fold change of 2.0 or greater.

In the present study, we performed an expression analysis of 2578 mature human miRNAs and identified 24 significantly upregulated miRNAs and 1 significantly downregulated miRNA in CM versus normal conjunctiva. Whereas several of these miRNAs have previously been described in cutaneous melanoma,^{15,16,18,21-31} none have been reported previously in UM samples.^{17,32,33} It was not possible to collect paired normal tissue for the comparison with CM, which may have limited the number of significant miRNAs identified in the present study.

We observed an upregulation of miR-20b-5p (miR-20b) in primary CM samples. This miRNA has previously been characterized as an oncomiR in cutaneous melanoma, and has been reported to be upregulated in primary and metastatic cutaneous melanoma.^{18,34} In another study, downregulation of miR-20b in metastatic cutaneous melanoma has been associated with activation of proteinase-activated receptor-1 (PAR-1), which is involved in tumor invasion and angiogenesis—and consequently tumor metastasis.^{15,35} Owing to this dual function of miR-20b, further investigations are needed to establish the prognostic and therapeutic potential of miR-20b in CM. Another interesting observation was the upregulation of miR-146a-5p (miR-146/miR-146a) and miR-146b-5p (miR-146b) in CM. Upregulation of miR-146a has been observed in primary and metastatic cutaneous melanoma, and it may be specific for metastatic disease.^{26,29,31} miR-146a promotes both initiation and progression of *BRAF/NRAS*-mutated cutaneous melanoma through increased activation of the NOTCH protein.²³ NOTCH1 is involved in melanoma formation and may enhance the metastatic potential of primary melanoma cells through activation of the mitogen-activated protein kinase (MAPK) pathway or the PI3K/Akt pathway.³⁶ Upregulation of miR-146b expression has consistently been reported in cutaneous melanoma and appears to be associated with melanoma progression.^{15,21,26,30} It is therefore evident that miR-146a and miR-146b have oncogenic roles in cutaneous melanoma, and these miRNAs may have a similar role in CM. The observed upregulation of miR-506-3p (miR-506) and miR-509-3p (miR-509) belonging to the miR-506-514 cluster has also been

described in metastatic cutaneous melanoma.^{15,16} In functional characterization of the miR-506-514 cluster, inhibition of the cluster has led to reduced cell growth and invasion and increased apoptosis in melanoma cell lines.¹⁶ Inhibition of these miRNAs may therefore be a new approach in the treatment of cutaneous melanoma and possibly CM.

To identify possible prognostic miRNAs, we analyzed the miRNA expression in TNM stages T1, T2, and T3 and compared the profiles with that for normal conjunctiva. We observed that upregulation of seven miRNAs shared by stage-T1 and stage-T2 CM divided the CM in clusters of low, intermediate, and high expression and that an increased expression was associated with increased tumor thickness, which is a known feature of poor prognosis in CM.³⁷⁻³⁹ One of the differentially expressed miRNAs specific for T1 and T2 tumors was miR-30d, which has been reported to have upregulated expression in metastatic cutaneous melanoma cell lines. Furthermore, upregulation of this miRNA correlates with increased tumor thickness, invasion, metastasis, and mortality in cutaneous melanoma patients.^{24,26} In addition, miR-30d targets several messenger RNAs involved in melanoma and/or cancer progression (*MITE1*, *ITGA5*, *SERPINE1*, and *ADAM19*).²²

Downregulation of miR-5096, which has not been reported previously in melanoma, was identified in stage-T2 tumors relative to normal conjunctiva. We did not identify differentially expressed miRNAs specific for T3 tumors, which may have been because of the small number of stage-T3 cases in the present study. To accurately detect stage-specific miRNAs, future studies should therefore include more T2 and T3 tumors.

The differences in tumor thickness observed may have been because the thinner tumors were more difficult to macrodissect. Thus, these samples may have had a higher proportion of normal tissue that interfered with the expression results. Microdissection of CM samples or the use of CM samples > 2 mm in thickness is therefore recommended for future studies.

Upregulation of two specific miRNAs (miR-3687 and miR-3916) was associated with a higher risk of local recurrence in CM patients. These miRNAs have not previously been described to be associated with melanoma development and/or progression. The lack of miRNAs significantly associated with metastasis or mortality may have been caused by the low number of patients developing metastasis and dying of melanoma-related causes in the present study.

Formalin-fixed, paraffin-embedded tumor samples are a readily available source of material for retrospective analyses. However, the degree of RNA degradation during the process of formalin fixation and its influence on miRNA expression levels have not been investigated in CM. Using tissue samples from

TABLE 4. Clinicopathologic Characteristics of Conjunctival ($n = 6$) and Other Head and Neck Mucosal Melanomas ($n = 4$) (Fresh Frozen Specimens)

| Case | Age, y | Sex | Melanoma Type | Location | Tumor Thickness, mm | TNM* |
|------|--------|-----|---------------|--|---------------------|------|
| 1 | 88 | F | Conjunctival | Epibulbar | 2 | T1 |
| 2 | 71 | F | Conjunctival | Extrabulbar | 3.5 | T2 |
| 3 | 49 | F | Conjunctival | Extrabulbar, eyelid skin | 1 | T3 |
| 4 | 34 | M | Conjunctival | Epibulbar | 10 | T1 |
| 5 | 81 | F | Conjunctival | Epibulbar | 3 | T1 |
| 6 | 92 | F | Conjunctival | Epibulbar | 5 | T1 |
| 7 | 76 | M | Mucosal | Nasal cavity | - | T4 |
| 8 | 87 | M | Mucosal | Nasal cavity, nasopharynx, maxillary sinus | - | T4 |
| 9 | 83 | M | Mucosal | Nasal cavity | - | T3 |
| 10 | 65 | F | Mucosal | Larynx | 10 | T3 |

Abbreviations: M, male; F, female.

* Tumors were classified according to the American Joint Commission on Cancer TNM classification (7th edition). None of the patients had nodal or distant metastatic disease at the time of diagnosis.

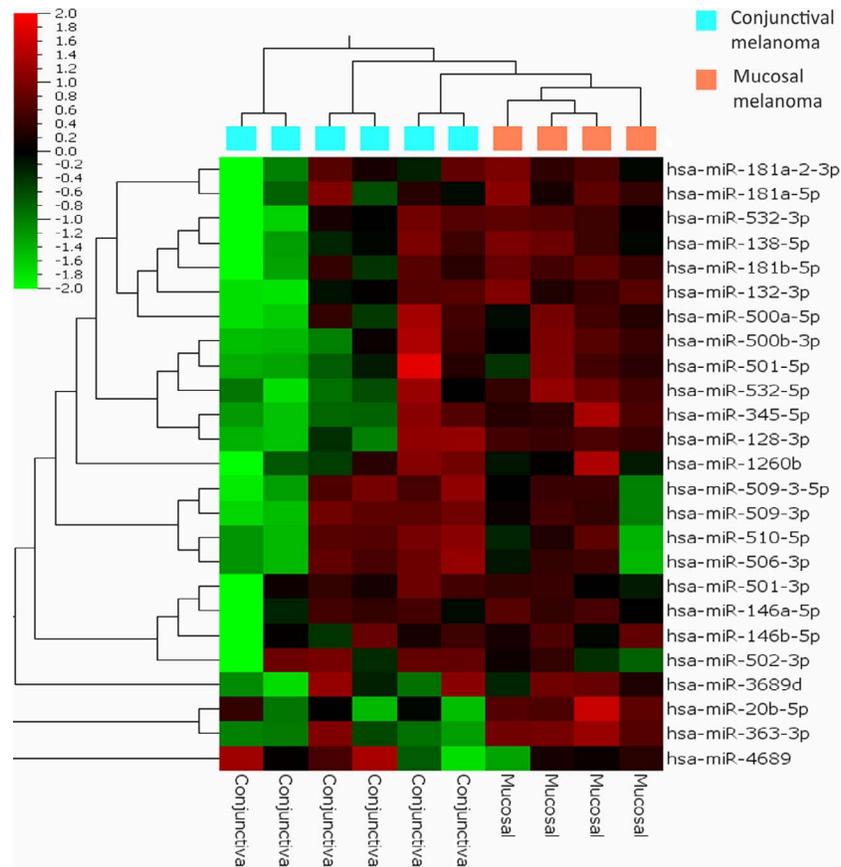


FIGURE 2. Hierarchical cluster visualization of 25 tumor-specific miRNAs identified in CM. The expression pattern of six CMs, three sinonasal mucosal melanomas, and one laryngeal mucosal melanoma was compared. No significant changes in expression were identified.

three patients, we were able to compare miRNA results from fresh frozen tissue and archived tissue from the same tumors. There was a good correlation between miRNA expression levels measured from FFPE CM samples and from fresh frozen CM samples, indicating that archived tissue is reliable for analysis of miRNA expression in CM.

Using fresh frozen tissue, we extended the study and investigated similarities in miRNA expression profiles between different subtypes of MM. No significant variation in expression of 25 tumor-specific miRNAs was observed between CM, sinonasal MM, and laryngeal MM. Owing to a low number of available fresh frozen tissue samples, this study was limited by the inclusion of mainly stage-T1 and stage-T2 CM, which was compared to stage-T3 and stage-T4 sinonasal and laryngeal melanoma. The subtypes of melanoma that we investigated all develop in mucosal membranes, and the miRNA expression may therefore also reflect shared embryologic or tissue-specific properties.

In conclusion, the novel finding in our study is a description of several miRNAs in CM. Many of these have previously been reported in cutaneous melanoma, and some may be potential prognostic biomarkers or possible future targets for therapy. Interestingly, no significant variation in the expression pattern of these miRNAs was observed when comparing different subtypes of MM. Thus, the miRNA expression in CM appears to be closely related to that of both cutaneous melanoma and other MMs. The miRNAs identified in the present study have not been described previously in CM and they warrant further investigation and validation.

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