Distinct Pathways in the Pathogenesis of Sebaceous Carcinomas Implicated by Differentially Expressed MicroRNAs

Michael T. Tetzlaff, MD, PhD; Jonathan L. Curry, MD; Vivian Yin, MD; Penvadee Pattanaprichakul, MD; Jane Manonukul, MD; Mongkol Uiprasertkul, MD; Ganiraju C. Manyam, MS; Khalida M. Wani, PhD; Kenneth Aldape, MD, PhD; Li Zhang, PhD; Victor G. Prieto, MD, PhD; Bita Esmaeli, MD

IMPORTANCE The molecular-genetic alterations contributing to the pathogenesis of sebaceous carcinoma and sebaceous adenoma remain poorly understood. Given that sebaceous carcinoma is associated with substantial morbidity and mortality, there is a critical need to delineate the pathways driving sebaceous carcinoma and candidate molecules for targeted therapy.

OBJECTIVE To describe differentially expressed microRNAs (miRNAs) in a series of periocular sebaceous carcinomas compared with sebaceous adenomas in order to identify pathways driving the pathogenesis of sebaceous carcinoma.

DESIGN, SETTING, AND PARTICIPANTS Thirty sebaceous carcinomas and 23 sebaceous adenomas (including 11 that were confirmed to be related to Muir-Torres syndrome and 6 that were confirmed to be sporadic) were obtained from archives (from 48 patients) of 2 institutions (University of Texas MD Anderson Cancer Center, Houston, and Siriraj Hospital, Mahidol University, Bangkok, Thailand) and profiled.

MAIN OUTCOMES AND MEASURES Expression of miRNAs was determined using total RNA from formalin-fixed, paraffin-embedded tissue and real-time reverse transcription–polymerase chain reaction performed in a microfluidics card containing 378 unique miRNAs. Fold change was determined using the ΔΔCt method (reference probe, RNU48). Median centering was used to normalize the data. Two-sample t tests were used to identify differentially expressed miRNAs. The false discovery rate was assessed by β-uniform mixture analysis of P values from the t statistics. Significance was defined by this estimated false discovery rate.

RESULTS Serial testing and validation confirmed overexpression of 2 miRNAs previously reported to be oncogenic, miR-486-5p (4.4-fold; P = 2.4 × 10−8) and miR-184 (3.5-fold; P = 1.7 × 10−6), in sebaceous carcinoma compared with sebaceous adenoma and downregulation of 2 miRNAs previously reported to have tumor-suppressive properties, miR-211 (−5.8-fold; P = 2.3 × 10−8) and miR-518d (−4.5-fold; 6.7 × 10−5), in sebaceous carcinoma compared with sebaceous adenoma.

CONCLUSIONS AND RELEVANCE Sebaceous carcinoma exhibits an miRNA expression profile distinct from that of sebaceous adenoma, implicating dysregulation of NF-κB and PTEN (targets of miR-486-5p) and TGF-β signaling (target of miR-211) in the pathogenesis of sebaceous carcinoma. The identification of miRNAs whose expression is altered in sebaceous carcinoma compared with sebaceous adenoma provides a novel entry point for a more comprehensive understanding of the molecular-genetic alterations pivotal to the development of sebaceous carcinoma.

Author Affiliations: Department of Pathology, University of Texas MD Anderson Cancer Center, Houston (Tetzlaff, Curry, Pattanaprichakul, Wani, Aldape, Prieto); Orbital Oncology and Ophthalmic Plastic Surgery, Department of Plastic Surgery, University of Texas MD Anderson Cancer Center, Houston (Yin, Esmaeli); Department of Dermatology, Siriraj Hospital, Mahidol University, Bangkok, Thailand (Pattanaprichakul, Manonukul, Uiprasertkul); Department of Biostatistics and Computational Biology, University of Texas MD Anderson Cancer Center, Houston (Manyam, Zhang).

Corresponding Author: Bita Esmaeli, MD, Orbital Oncology and Ophthalmic Plastic Surgery, Department of Plastic Surgery, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Unit 1488, Houston, TX 77030 (besmaeli@mdanderson.org).
Sebaceous carcinoma of the eyelid is a rare disease that accounts for approximately 1% of all eyelid tumors and 5% of all malignant epithelial eyelid tumors.\(^1\)\(^-\)\(^5\) Sebaceous carcinoma and its benign counterpart, sebaceous adenoma, are cutaneous markers of microsatellite instability in some patients with Muir-Torre syndrome. Risk factors for sebaceous carcinoma include immune deficiency, retinoblastoma, and prior irradiation. Sebaceous carcinoma typically exhibits aggressive local behavior and tends to recur locally and metastasize to regional lymph nodes and distant organs.\(^1\)\(^,\)\(^6\)\(^,\)\(^7\) Orbital exenteration is necessary in 13% to 23% of cases.\(^5\)\(^,\)\(^7\)\(^,\)\(^8\) Local or distant metastasis is estimated to occur in 8% to 22% of patients with sebaceous carcinoma at some point in their disease course, and the estimated percentage of patients with sebaceous carcinoma who die of the disease ranges from 6% to 10% and could be as high as 22%.\(^7\)\(^-\)\(^9\)

Thus, despite a tendency for locally aggressive behavior (often necessitating debilitating surgery), frequent regional and distant metastasis, and death due to disease, comparatively little is known about the molecular pathogenesis of sebaceous carcinoma. There is therefore a critical need to identify the pathways driving sebaceous carcinoma in order to identify potential candidate molecules for targeted therapy to minimize the morbidity and mortality due to this disease.

**MicroRNAs (miRNAs)** are approximately 20- to 24-nucleotide, highly conserved noncoding RNA molecules that function mostly in posttranscriptional gene regulatory pathways by binding to target messenger RNA and initiating either translational repression or cleavage.\(^10\)\(^,\)\(^11\) Cancer-associated genomic regions are portions of the genome that exhibit a heightened susceptibility to structural alterations in cancer. The demonstration that more than 50% of miRNA genes localize to cancer-associated genomic regions predicted global derangements of miRNA expression in human malignancies.\(^12\) This prediction has been fulfilled: alterations in miRNA expression have been described in virtually all human cancer types. These efforts are significant to the extent that miRNAs with significantly different expression in tumors vs normal/benign tissue provide a critical window to biological pathways hijacked during tumorigenesis and/or metastasis. MicroRNA signatures also serve as instructive molecular surrogates, which are exploited to distinguish benign from malignant tissue and to predict clinical outcome in many cancer types.\(^12\)\(^-\)\(^22\) Finally, because of their small size and relative stability in formalin-fixed, paraffin-embedded tissues, miRNAs offer an important advantage over other, less robust molecular analytes (eg, protein, DNA, and messenger RNA) for which fresh frozen tissue is the gold standard for characterization.\(^23\) The stability of miRNAs is particularly important in the case of retrospective studies, for which formalin-fixed, paraffin-embedded tissue is often the only resource available for molecular analyses.

Herein, we compared the miRNA expression profiles of 30 specimens of sebaceous carcinoma obtained from 26 patients and 23 specimens of sebaceous adenoma obtained from 22 patients. We found significant overexpression of 2 miRNAs previously reported to be oncogenic, miR-486-5p and miR-184, and significant downregulation of 2 miRNAs previously reported to have tumor-suppressive function, miR-211 and miR-195, in sebaceous carcinoma compared with sebaceous adenoma. Our results implicate dysregulation of the pathways targeted by these miRNAs as possible targets for rationally designed therapies.

**Methods**

**Tissue Samples**

Tissue samples obtained from patients with a diagnosis of either sebaceous adenoma or sebaceous carcinoma during the period from 2003 to 2012 were collected from archives in the Department of Pathology and Laboratory Medicine, the University of Texas MD Anderson Cancer Center, Houston, and Siriraj Hospital, Mahidol University, Bangkok, Thailand. All tissues were collected from the archives according to the guidelines and policies and with the approval of the institutional review boards of MD Anderson Cancer Center and Siriraj Hospital. A material transfer agreement between the 2 institutions was obtained, and subsequent studies on selected tissues were performed at MD Anderson Cancer Center. Hematoxylin-eosin–stained sections were reviewed, and diagnoses were confirmed for our study by 2 dermatopathologists (M.T.T. and J.L.C.). The data were deidentified. We obtained 30 specimens of sebaceous carcinoma (10 from MD Anderson Cancer Center and 20 from Siriraj Hospital) from 26 patients. These specimens included paired specimens of the primary tumor and locally recurrent disease obtained from 2 patients and paired specimens of the primary tumor and a lymph node metastasis obtained from 2 patients. We obtained 23 clinically distinct specimens of sebaceous adenoma (17 from MD Anderson Cancer Center and 6 from Siriraj Hospital) from 22 patients. None of these 23 specimens were specimens of recurrent disease. These specimens included 6 lesions known to be sporadic lesions and 11 Muir-Torre syndrome–associated lesions. Ten to twenty 10-μm sections per sample were obtained from the selected block, and the area of interest was delineated by 2 dermatopathologists (M.T.T. and J.L.C.) and macrodissected from a glass slide for the extraction of total miRNA.
RNA Isolation and miRNA Profiling

Total RNA was extracted from formalin-fixed, paraffin-embedded tissue using the Epicenter RNA isolation kit (Epicenter Biotechnologies). RNA quality was confirmed, and total RNA was reverse transcribed, using an ABI Taqman miRNA reverse transcription kit (Applied Biosystems). Real-time polymerase chain reaction (Applied Biosystems) was performed in a microfluidics card containing 378 unique miRNAs.

Statistical Analyses

Data were analyzed using R statistical computing software. To avoid underflow of fold changes, the lower bound of the detection limit for −ΔCt values was set at −37. −ΔCt values that were less than −37 were set at −37. The reference probe “RNU48-001006” was used to calculate the −ΔCt values, which represented miRNA expression levels. The −ΔCt values were further normalized by median-centering each sample.

The miRNA expression data used in our study were generated in 2 batches. Because there was a strong batch effect between the 2 data sets, the ComBat empirical Bayesian method was used to correct the batch effect. The P values of significance of differential expression were based on r tests of −ΔCt values between the sebaceous carcinoma group and the sebaceous adenoma group, and estimation of the false discovery rates was based on the β-uniform mixture model.

Results

Patient Demographics

The demographic characteristics of the patients and the tumor locations for the cases studied are summarized in Table 1. The median age was 72 years (range, 31-94 years) for the patients with sebaceous carcinoma and 62 years (range, 47-88 years) for the patients with sebaceous adenoma. All of the sebaceous carcinomas except 1 arose in a periocular location, and our cohort of sebaceous carcinoma specimens included 2 lymph node metastases. Among the sebaceous adenomas, 16 arose on the head and neck, and 6 arose on the trunk.

miRNAs With Significantly Different Expression in Sebaceous Carcinoma and Sebaceous Adenoma

We performed serial testing and validation miRNA profiling experiments on a total of 53 specimens of sebaceous neoplasms. Unsupervised hierarchical clustering analysis of these 53 sebaceous neoplasms according to their global patterns of miRNA expression revealed essentially 2 distinct clusters of lesions, each of which contained relatively distinct groups of sebaceous carcinoma and sebaceous adenoma (Figure 1). A total of 7 sebaceous carcinomas and 7 sebaceous adenomas were misclassified according to the whole pool of miRNAs profiled among the 53 cases we studied. In addition, with the exception of the specimens from 1 patient (whose primary biopsy was classified as adenoma, while the recurrent lesion clustered together with the carcinomas), all of the paired specimens of primary tumors and recurrent/metastatic lesions obtained from patients with sebaceous carcinoma clustered together quite closely on the dendrogram (Figure 1).

To identify miRNAs whose expression was most robustly different between the 2 groups, we applied a minimum threshold of a false discovery rate of less than 0.01 and at least a 2-fold change in expression. According to these thresholds, we identified 20 miRNAs whose expression in sebaceous carcinoma was significantly different from that in sebaceous adenoma (Table 2). Of particular interest, we found overexpression of 2 miRNAs previously reported to be oncogenic, miR-486-5p (4.4-fold; P = 2.4 × 10−8) and miR-184 (3.5-fold; P = 1.7 × 10−4), and downregulation of 2 miRNAs previously reported to have tumor-suppressive properties, miR-211 (−5.8-fold; P = 2.3 × 10−5) and miR-518d (−4.5-fold; 6.7 × 10−5), in sebaceous carcinoma compared with sebaceous adenoma. These clustering analyses did not demonstrate miRNA expression profile differences in the relationship among sebaceous adenomas (sporadic vs Muir-Torre syndrome–associated) (Figure 1 and Figure 2). In separate focused analyses, we did not observe any significant differentially expressed miRNAs among the known sporadic sebaceous adenomas compared with those arising in patients with confirmed Muir-Torre syndrome (data not shown).

Next, we performed a supervised hierarchical clustering analysis using only the 20 miRNAs with significantly different expression in sebaceous carcinoma compared with sebaceous adenoma (Figure 2) and found that, according to the relative expression levels of these 20 miRNAs, all but 5 of the tumor specimens (1 carcinoma and 4 adenomas) were correctly classified as either carcinoma or adenoma. According to the relative expression of those 20 miRNAs whose expression was significantly different in sebaceous carcinoma compared with sebaceous adenoma, all of the paired specimens of primary tumors and recurrent/metastatic lesions from patients with sebaceous carcinoma clustered together quite closely on the dendrogram (Figure 2).
Figure 1. Unsupervised Hierarchical Clustering Analysis of Sebaceous Neoplasms According to All MicroRNAs (miRNAs) Profiled

<table>
<thead>
<tr>
<th>miRNA</th>
<th>P Value</th>
<th>t Statistic</th>
<th>Fold Change</th>
<th>Mean Expression Carcinoma</th>
<th>Mean Expression Adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-486-5p</td>
<td>2.4 × 10^{-8}</td>
<td>-6.60</td>
<td>4.42</td>
<td>0.98</td>
<td>-3.44</td>
</tr>
<tr>
<td>hsa-miR-363</td>
<td>3.7 × 10^{-10}</td>
<td>-7.74</td>
<td>4.35</td>
<td>1.44</td>
<td>-2.91</td>
</tr>
<tr>
<td>hsa-miR-133b</td>
<td>1.2 × 10^{-5}</td>
<td>-4.85</td>
<td>4.21</td>
<td>1.97</td>
<td>-2.24</td>
</tr>
<tr>
<td>hsa-miR-133a</td>
<td>2.1 × 10^{-5}</td>
<td>-4.68</td>
<td>3.92</td>
<td>1.97</td>
<td>-1.95</td>
</tr>
<tr>
<td>hsa-miR-1</td>
<td>7.1 × 10^{-5}</td>
<td>-4.33</td>
<td>3.60</td>
<td>1.40</td>
<td>-2.20</td>
</tr>
<tr>
<td>hsa-miR-184</td>
<td>1.7 × 10^{-6}</td>
<td>-5.41</td>
<td>3.45</td>
<td>2.10</td>
<td>-1.35</td>
</tr>
<tr>
<td>hsa-miR-486-3p</td>
<td>2.0 × 10^{-5}</td>
<td>-4.71</td>
<td>3.04</td>
<td>1.74</td>
<td>-1.30</td>
</tr>
<tr>
<td>hsa-miR-503</td>
<td>1.5 × 10^{-5}</td>
<td>-4.79</td>
<td>2.65</td>
<td>1.02</td>
<td>-1.63</td>
</tr>
<tr>
<td>hsa-miR-324-5p</td>
<td>8.0 × 10^{-5}</td>
<td>-4.29</td>
<td>2.61</td>
<td>0.97</td>
<td>-1.64</td>
</tr>
<tr>
<td>hsa-miR-34c</td>
<td>1.5 × 10^{-4}</td>
<td>-4.10</td>
<td>2.61</td>
<td>0.81</td>
<td>-1.80</td>
</tr>
<tr>
<td>hsa-miR-135b</td>
<td>2.4 × 10^{-6}</td>
<td>-5.31</td>
<td>2.40</td>
<td>0.94</td>
<td>-1.46</td>
</tr>
<tr>
<td>hsa-miR-302c</td>
<td>1.8 × 10^{-3}</td>
<td>-3.30</td>
<td>2.31</td>
<td>1.50</td>
<td>-0.81</td>
</tr>
<tr>
<td>hsa-miR-15a</td>
<td>2.8 × 10^{-3}</td>
<td>-3.14</td>
<td>2.23</td>
<td>0.65</td>
<td>-1.58</td>
</tr>
<tr>
<td>hsa-miR-542-3p</td>
<td>1.5 × 10^{-4}</td>
<td>-4.10</td>
<td>2.21</td>
<td>0.97</td>
<td>-1.25</td>
</tr>
<tr>
<td>hsa-miR-301</td>
<td>2.6 × 10^{-5}</td>
<td>-4.63</td>
<td>2.17</td>
<td>1.00</td>
<td>-1.17</td>
</tr>
<tr>
<td>hsa-miR-597</td>
<td>1.6 × 10^{-3}</td>
<td>-3.34</td>
<td>2.15</td>
<td>0.45</td>
<td>-1.70</td>
</tr>
<tr>
<td>hsa-miR-135a</td>
<td>1.0 × 10^{-3}</td>
<td>-3.48</td>
<td>2.02</td>
<td>0.54</td>
<td>-1.48</td>
</tr>
<tr>
<td>hsa-miR-196b</td>
<td>4.9 × 10^{-3}</td>
<td>2.95</td>
<td>-2.07</td>
<td>-0.26</td>
<td>1.81</td>
</tr>
<tr>
<td>hsa-miR-518d</td>
<td>6.7 × 10^{-5}</td>
<td>4.34</td>
<td>-4.51</td>
<td>-1.33</td>
<td>3.18</td>
</tr>
<tr>
<td>hsa-miR-211</td>
<td>2.3 × 10^{-9}</td>
<td>7.24</td>
<td>-5.81</td>
<td>-3.06</td>
<td>2.75</td>
</tr>
</tbody>
</table>

*Selected miRNAs derived after application of a minimum threshold of a false discovery rate of less than 0.01 and a fold change of 2.0 or greater.
Confirmation of miR-486 Upregulation in Sebaceous Carcinoma by In Situ Hybridization

Figure 3A shows the relative expression levels of miR-486-5p in the sebaceous carcinomas and sebaceous adenomas analyzed in our study. To confirm that miR-486-5p is indeed overexpressed in the tumor cells of sebaceous carcinoma, we performed in situ hybridization on a subset of cases of sebaceous carcinoma. Our findings confirmed expression of miR-486-5p in benign epithelial cells of the skin and conjunctiva (the epidermis, conjunctival mucosa, and adnexal structures), including benign sebaceous glands (Figure 3B and C). In particular, in situ hybridization confirms nuclear and cytoplasmic expression of miR-486-5p in benign sebaceous glands. Whereas benign sebaceous glands exhibited relatively low levels of expression of miR-486-5p, the tumor cells comprising sebaceous carcinoma exhibited strong and diffuse expression (both nuclear and cytoplasmic expression, but with strongly accentuated cytoplasmic expression) of miR-486-5p, confirming upregulation of this miRNA in sebaceous carcinoma (Figure 3D and E). Essentially identical findings were seen for miR-184 (data not shown).

Discussion

Herein, we determined the miRNA expression profiles of 53 sebaceous neoplasms, including 30 sebaceous carcinomas and 23 sebaceous adenomas. We found overexpression of 2 miRNAs previously reported to be oncogenic, miR-486-5p and miR-184, and downregulation of 2 miRNA previously reported to have tumor-suppressive properties, miR-211 and miR-195, in sebaceous carcinoma compared with sebaceous adenoma. We confirmed the dysregulation of miR-486-5p and miR-184 in sebaceous carcinoma tumor cells by in situ hybridization. To the best of our knowledge, the present study represents the first large-scale study describing altered miRNA expression profiles in sebaceous tumors. Our findings represent a critical entry point into a greater mechanistic understanding of sebaceous carcinogenesis to the extent that they implicate dysregulation of the pathways targeted by these miRNAs.

Prior studies have shown that miR-486 functions as an oncogene in human glioma by inhibiting the translation of multiple NF-κB-negative regulators, thereby driving constitutive activation of NF-κB signaling cascades. Li and
Akt) pathway, which promotes entry into the cell cycle.\textsuperscript{29-33} phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/tensoin homologue (PTEN), a negative regulator of the Akt pathway and a key factor in the development of pathologic changes in studies characterizing the spectrum of molecular-genetic changes underlying Duchenne muscular dystrophy.\textsuperscript{29} Enforced expression of miR-486 in mouse skeletal muscle resulted in reduced levels of PTEN and increased levels of phosphorylated Akt.\textsuperscript{30} Furthermore, miR-486 is upregulated in malignant T cells in patients with Sézary syndrome, where miR-486 confers resistance to pro-apoptotic pathways.\textsuperscript{34} Subsequently, miR-486 was shown to regulate PTEN expression levels in these T cells.\textsuperscript{31} Together, these findings highlight PTEN as an additional potential tumor suppressor targeted by miR-486-5p in sebaceous carcinoma. Furthermore, miR-486 exerts an oncogenic role in acute myeloid leukemia arising in patients with Down syndrome compared with patients with sporadically acquired acute megakaryocytic leukemia.\textsuperscript{35} However, a specific oncogenic role for miR-486 in cancer has not been proven in all cancer types; miR-486 functions as a tumor suppressor in hepatocellular carcinoma,\textsuperscript{36} lung carcinoma,\textsuperscript{37,38} breast carcinoma,\textsuperscript{39} myxoid liposarcoma,\textsuperscript{40} and gastric carcinoma.\textsuperscript{41}

A number of studies have shown miR-184 to be a potent oncogenic miRNA in oral squamous cell carcinoma (SCC). Overall, upregulation of miR-486 in human gliomas compared with adjacent brain tissue;\textsuperscript{27} (2) showed that progressively increased expression of miR-486 correlated with increased World Health Organization glioma grade, and (3) showed that miR-486 directly represses numerous negative regulators of NF-κB.\textsuperscript{28} These findings indicate that upregulation of miR-486 in gliomas results in unchecked activation of NF-κB-driven pathways. An additional target of miR-486-5p is phosphatase and tensin homologue (PTEN), a negative regulator of the PI3K pathway.\textsuperscript{44} In malignant gliomas, miR-184 expression is downregulated compared with controls, and plasma miR-184 levels decreased following surgical extirpation. Finally, abrogation of miR-184 expression in cells lines of SCC of the tongue resulted in impaired cell proliferation, which correlated with downregulation of c-Myc and increased apoptosis.\textsuperscript{42} However, as has been shown for other candidate oncogenic miRNAs, miR-184 also functions as a canonical tumor suppressor in some tumors. miR-184 expression diminishes with progression of diffuse astrocytoma to anaplastic astrocytoma and secondary glioblastoma, and enforced expression of miR-184 in glioma cells resulted in reduced proliferation, apoptosis, and cell invasion in vitro.\textsuperscript{43} In neuroblastoma, amplification of the MYC transcription factor results in diminished miR-184 expression, relieving miR-184-mediated repression of AKT2 and culminating in activation of the PI3K pathway.\textsuperscript{44} In malignant gliomas, miR-184 expression inversely correlates with the expression of its target, SNDI. Enforced expression of miR-184 in glioma cells suppressed cell invasion, colony formation, and anchorage-independent growth in soft agar, and enforced expression of miR-184 in mouse xenografts resulted in tumors with reduced invasion and improved survival.\textsuperscript{45} In non-small cell lung carcinoma, the tumor suppressor CDC19 activates miR-184, which in turn suppresses c-Myc, impairing PI3K/Akt-mediated cell proliferation.\textsuperscript{46} Enforced expression of miR-184 in lung carcinoma cells resulted in repressed cell proliferation, which correlated with reduced expression of cyclin D1 and increased expression of the cell cycle inhibitor p21.

In melanoma progression, miR-211 functions as a tumor suppressor: its expression is downregulated compared with benign melanocytic nevi. Enforced expression of miR-211 in melanoma cell lines inhibits anchorage-independent colony formation and invasion.\textsuperscript{47,48} miR-211 negatively impacts TGF-β
Differentially Expressed miRNAs in Sebaceous Carcinoma vs Adenoma

Original Investigation Research

Herein, we demonstrated dysregulation of discrete miRNAs in sebaceous carcinoma vs sebaceous adenoma, including miR-486-5p, miR-184, and miR-211. Additional studies are ongoing to determine the precise targets of these miRNAs in sebaceous carcinoma. Identification of these targets will present a novel opportunity to devise targeted treatment strategies for this rare but aggressive malignancy.

REFERENCE


33. Narducci MG, Ardeli D, Picchio MC, et al. MicroRNA profiling reveals that miR-21, miR486


