Oncogenic Function and Early Detection Potential of miRNA-10b in Oral Cancer as Identified by microRNA Profiling

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Abstract
The miRNA participates in a variety of biologic processes, and dysregulation of miRNA is associated with malignant transformation. In this study, we determined specific profile of miRNA associated with oral cancer by using miRNA array screening method. There were 23 miRNAs found with considerably differential expressions between six oral cancer cell lines and five lines of normal oral keratinocytes, in which, 10 miRNAs showed the highest significant difference after independent examination by reverse transcription quantitative PCR. Eight molecules were upregulated, miR-10b, miR-196a, miR-196b, miR-582-5p, miR-15b, miR-301, miR-148b, and miR-128a, and two molecules, miR-503 and miR-31, were downregulated. The most upregulated miR-10b was further examined, and its functions were characterized in two oral cancer cell lines. The miR-10b actively promotes cell migration (2.6- to 3.6-fold) and invasion (1.7- to 1.9-fold) but has minimal effect on cell growth or chemo-/radiosensitivity. Furthermore, miR-10b was considerably elevated in the plasma of xenografted tumor mice (20-fold). This upregulation of miR-10b in plasma was further shown in the patients with oral cancer [P < 0.0001, area under curve (AUC) = 0.932] and precancer lesions [P < 0.0001, AUC = 0.967], suggesting that miR-10b possesses a high potential to discriminate the normal subjects. In conclusion, we have identified at least 10 miRNAs significantly associated with oral cancer, including the most elevated miR-10b. The miR-10b actively participates in cancer formation by promoting cell migration and invasion. Our study using clinical samples suggests that plasma miR-10b has high potential as an early detection marker for oral cancer. Cancer Prev Res; ©2012 AACR.

Impact of this paper:
1. miRNA signature of oral cancer was determined.
2. The oncogenic function of miR-10b in oral cancer was first demonstrated.
3. The potential of miR-10b as a circulating biomarker for the early detection of oral cancer was presented.

Introduction
Oral cancer is one of the most common cancers worldwide, with an incidence estimated to be more than 500,000 annually (1, 2). The disease is more prevalent among males than females (1, 2). Epidemiologic studies have shown a strong association between oral cancer and environmental carcinogens, especially the use of tobacco, alcohol, and betel quid (3, 4). The 5-year mortality rate for oral cancer has not been altered significantly in the last few decades, despite advances in treatment modalities. Even if there is a good treatment response, patients with advanced disease often suffer from substantial functional and cosmetic morbidity, which decreases their quality of life. The genetic alterations leading to oral carcinogenesis remain to be identified.

The miRNAs are endogenous, small noncoding RNAs (18–25 nucleotides long) that negatively regulate gene expression at the translational level by base pairing to the 3′-untranslated region of target mRNAs (5, 6). There are now more than 700 human miRNAs annotated in the Sanger miRBase database, and it has been predicted that there are more than 1,000 total human miRNAs (6, 7). It is estimated that approximately one third to one half of human genes are regulated by miRNAs, and each miRNA is predicted to target several hundred transcripts, making miRNAs one of the biggest family of gene regulators. Because miRNAs participate in a variety of biologic processes, including cell proliferation, differentiation, apoptosis, and migration, it is conceivable that the dysregulation...
of miRNAs is associated with malignant transformation. Recently, large-scale miRNA screening that shows unique expression profiles has been conducted for several types of cancer. In head and neck cancer, miRNA expression also has been screened (8–12); however, few overlapping molecules were found between profiling studies, indicating that the complexity and underlying mechanisms of miRNA function have not been solved. For example, sample profiling that was derived from a specific anatomic subsite (11) or examination using a different design or methodology (8–11) might lead to a different outcome. Moreover, as head and neck cancer is strongly associated with environmental carcinogens, reports from different geographic areas may lead to different results (10–12).

In the present study, we profiled and compared the miRNA of oral cancer in areas prevalent for betel quid chewing using 6 oral cancer lines and 5 normal keratinocyte cell lines. We identified 23 miRNAs and confirmed differential expression in 10 of them. We further examined the cellular function and plasma levels of miR-10b and showed its highly diagnostic potential for early detection in the plasma of oral cancer.

Materials/Subjects and Methods

Cells and cell lines
A total of 6 oral cancer lines, SCC25 (13), SAS (14), OECM1 (15), OEC3 (16), CGHNC8, and CGHNC9, and 5 normal keratinocyte cell lines, CGHNK2, CGHNK4, CGK1, CGK5, and CGK6, were used. SCC25 cell line was purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan. SAS, OECM1, and OEC3 cell lines were kindly distributed by Professors S.C. Lin and K.W. Chang, Yang Ming University, Taipei, Taiwan. CGHNC8 and CGHNC9 cell lines were established within recent 2 years, derived from oral cancer squamous cell carcinomas of patients from Chang Gung Memorial Hospital, Taoyuan, Taiwan. The normal keratinocytes CGHNK2, CGHNK4, CGK1, CGK5, and CGK6 were primary culture cells from tissue biopsies of grossly normal oral mucosa in recent 2 years, with (CGHNK2, CGHNK4) or without (CGK1, CGK5, and CGK6) human papilloma virus (HPV) immortalization (Chang Gung Memorial Hospital). All the cell lines established in Taiwan (OECM1, OC3, CGHNC8, CGHNC9, CGHK2, CGHNK4, CGK1, CGK5, and CGK6) were derived from patients with oral cancer with the habits of areca chewing and smoking, except two without areca chewing (CGHNK2 and CGK1) and one without smoking (OC3). Cell lines SCC25 (13), SAS (14), OECM1 (15), and OC3 (16) were originally authenticated using the AmpFlSTR® (Applied Biosystems), which contains 13 human microsatellites, to analyze the expression level of miRNAs from the microarray. Total RNA was isolated from cells with TRIzol reagent (Gibco BRL). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ air.

Reverse transcription quantitative PCR analysis for miRNA validation
Total RNA was isolated from cells with TRizol reagent (Gibco BRL). Reverse transcription was conducted as previously described (18), except for the use of miRNA specific stem-loop RT primers. TaqMan miRNA assays kits (ABI) were used to examine specific miRNA expression by reverse transcription quantitative PCR (RT-PCR), according to the manufacturer's suggested protocol. The real-time PCR results, recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6 RNA), and the comparative threshold cycle method (ΔCt) was used to determine miRNA expression.

Transfection of miRNA antagonomir oligonucleotide
Cells were seeded for transfection at a density of 5 × 10^5 in a 100-mm dish and cultured for 16 hours. When 50% confluence was reached, cells were transfected with various concentrations (75–900 μmol/L) of mir-10b antagonomir or scramble oligonucleotides, by using Lipofectamine 2000 reagent (Invitrogen) in OPTI-MEM medium (Invitrogen) for 16 hours; then, the medium was replaced with fresh complete medium. The nucleotide sequence of mir-10b antagonomir was 5’-CACAAATTCGTTCTACAGCCATA-3’.

The scramble oligonucleotides contained the same
nucleotides as the miR-10b antagonist but in random sequence, 5’ CTG TTC GCA CAG CTT GGT TAA AA-3’.

**Colony formation assay**

To determine colony-forming ability, cells were transfected with either antagonist or scramble oligonucleotides. A total of 1,000 cells were seeded in 6-well plates and allowed to grow for 7 days without disruption. After 70% ethanol fixation, the plate was stained with 0.5% crystal violet, and cell colonies were counted.

**Cell migration assay**

Cell migration was evaluated by an in vitro wound-healing assay. After transfection with either miR-10b antagonist or scramble oligonucleotides, 70 μL of 5 × 10^5 cell/mL transfectants was seeded in an ibidi culture insert (Applied BioPhysics, Inc.) on top of a 6-well plate. After 8 hours of incubation, the culture insert was detached to form a cell-free gap in a monolayer of cells. After changing to culture medium with 1% fetal calf serum, the cell migration status toward the gap area was photographed every 6 hours.

**Matrigel invasion assay**

The cell invasion assay was conducted using BioCoat Matrigel (Becton Dickinson Biosciences) and Millicell invasion chambers (Millipore) similar to those previously described (19). Briefly, Matrigel was coated onto the membrane of the Millicell upper chamber with a pore size of 8 μm in a 24-well plate for 12 hours at 37°C. After transfection with either antagonist or scramble oligonucleotides, cells were seeded into the upper chamber at a density of 1 × 10^5 cells per well in 0.2 mL of 1% FBS medium. The lower chamber contained complete culture medium, which included 10% FBS to attract invading cells. Cells were incubated at 37°C for 24 hours, and the number of cells that invaded through the Matrigel-coated membranes was counted and compared with the number of cells that passed through the membrane in the control chambers. The invaded cells on lower side of membrane were fixed, stained with crystal violet, and photographed.

**Determination of chemo- or radiosensitivity**

Cells, either transfected with antagonist or scramble oligonucleotides, were seeded at 1 × 10^5 cells per well in 6-well plates for 8 hours. For determination of chemosensitivity, cells were treated with various doses (0–80 μg/mL) of cisplatin and continuously cultured for 2 days. The number of surviving cells was counted and compared with the number of surviving untreated cells. For determination of radiosensitivity, the 1,000 antagonist or scramble oligonucleotide transfected cells were seeded into a 6-well cell culture plate. After incubation for 8 hours, cells were exposed to a range of radiation doses (0–80 Gy) and continuously cultured for 7 days, followed by the calculation of the surviving colonies. The survival fraction was calculated as the number of colonies divided by the number of cells seeded times plating efficiency, as previously described (15).

**Patients and the determination of plasma miRNA**

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, and written informed consent was obtained from all participants. The EDTA plasma samples were collected from 54 patients with oral cancer in the week before receiving operation and/or chemoradiotherapy. The characteristics of these patients with oral cancer are summarized in the Table 1. These included 51 (94%) males and 3 (6%) females, with a mean age of 52.0 years. The EDTA plasma samples from 36 age- and sex-matched normal individuals and 7 patients with oral precancer leukoplakia were also obtained to compare. The miRNA of each specimen was purified from 200 μL plasma using the miRNeasy Mini Kit (Qiagen Inc.) and dissolved in 20 μL of RNase-free water. The miRNA levels

| Table 1. Clinical characteristics of patients recruited in this study |
|-------------------------|---------|--------|--------|
| Item                    | Cancer  | Precancer | Normal |
| Sex                     | Male    | Female  | Male    | Female  |
|                         | 51 (94%)| 3 (6%)  | 7 (100%)| 0 (0%)  |
| Age                     | Mean ± SD | 52.0 ± 12.8 | 51.9 ± 10.2 | 50.5 ± 12.6 | 52.0 ± 12.8 |
|                         | <40 y   | 11 (20%) | 1 (14%) | 6 (17%) |
|                         | 41–50 y | 15 (28%) | 2 (29%) | 11 (31%) |
|                         | 51–60 y | 14 (26%) | 2 (29%) | 12 (34%) |
|                         | ≥61 y   | 14 (26%) | 2 (29%) | 6 (17%) |
| Habits                  | Alcohol drinking | 39 (73%) | 2 (29%) |
|                         | Betel quid chewing | 40 (74%) | 2 (29%) |
|                         | Cigarette smoking | 42 (78%) | 6 (86%) |
| T stage                 | T1–T2   | 31 (57%) |
|                         | T3–T4   | 23 (43%) |
| N stage                 | 0       | 30 (56%) |
|                         | 0       | 24 (44%) |
| Overall stage           | III–IV  | 34 (63%) |
| Pathologic diagnosis    | Squamous hyperplasia | 4 (57%) |
|                         | Verrucous hyperplasia | 1 (14%) |
|                         | Hyperkeratosis | 2 (29%) |
| Total                   | 54 (100%) | 7 (100%) | 36 (100%) |
were determined by RT-qPCR using the method described above.

**Mice and xenograft tumors**

To determine whether plasma miR-10b levels could serve as a diagnostic marker, we established a xenograft oral cancer SAS tumor in BALB/C nude mice. Five-week-old male BALB/c null mice, cared for according to institutional guidelines, were used for the study. For the xenograft in situ study, mice were injected subcutaneously in the upper portion of the hind limb with $1 \times 10^6$ SAS cells. Mice were then monitored for the tumor growth by calculating the size of the tumor's length $\times$ width $\times$ height after 6 weeks. The EDTA plasma was drawn before cancer cell injection and 6 weeks after xenografting. The level of plasma miR-10b was determined by RT-qPCR as described above.

**Statistical analysis**

The $\chi^2$ t test was used to determine whether miRNA levels were different between the two groups of samples. A $P$ value of less than 0.05 was considered statistically significant. To evaluate whether RT-qPCR data could separate differences between normal individuals and patients with cancer, receiver operating characteristic (ROC) analysis was conducted, and the area under curve (AUC) was used to measure the level of separation between the 2 groups.

**Results**

**Expression profiles of miRNA between oral cancer cells and normal keratinocytes**

An miRNA microarray (Agilent Technology) was used to globally profile miRNAs that are differentially expressed between 6 oral cancer cells and 5 lines of normal keratinocytes. The mean signal intensity of the cancer cell lines versus normal keratinocytes is shown in Fig. 1A. After normalization and filtering to exclude weak signals, 190 miRNAs were selected for clustering analysis. Unsupervised hierarchical clustering analysis and the ANOVA were used to find differentially expressed miRNAs. With an FDR $< 0.1$ and more than a 2-fold change between the cancerous and the normal group, 23 miRNAs were found of which 19 were upregulated and 4 downregulated in the cancer samples (Fig. 1B, Table 2). The expression trends of these miRNAs were homogenous across almost all samples in the same group, with low $P$ values (all $P < 0.05$, except miR-196a and miR-638, which were marginal). miR-10b, miR-9, miR-196a, and miR-196b showed high overexpression (>10-fold). These results suggest that these 23 miRNAs may play important roles in oral carcinogenesis.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Hierarchical clustering analysis of miRNAs between cancer cell lines and normal keratinocytes derived from oral tissues. A total of 11 samples, including 6 oral cancer cell lines and 5 lines of normal oral keratinocytes, were analyzed with a 470 miRNA-based microarray assay (Agilent Technology). A, the mean intensities of 470 miRNAs in 5 normal oral keratinocytes (x-axis) versus 6 oral cancer cell lines (y-axis) are shown. Intensity below 100 was considered as a weak signal. B, hierarchical cluster analysis to show the similarity of 23 miRNAs between cancer and normal groups. After filtering out the weak signals in the samples, 190 miRNAs were selected. Using ANOVA with FDR $< 0.1$ and a greater than 2-fold change of expression as selection criteria, 23 miRNAs were found to have significantly different expression levels between normal and cancerous cells.
RT-qPCR analysis for 23 miRNAs

To further assess the significance of these miRNAs in oral cancer, RT-qPCR assays were independently examined between 6 oral cancer cells and 5 lines of normal keratinocytes. For each miRNA, the results are summarized in the Supplementary Table S1. Figure 2A shows representative miRNAs that were significantly differentially expressed between cancer and normal cells (also see in Supplementary Fig. S1). There were 20 miRNAs confirmed to have more than a 1.5-fold change in expression and 10 miRNAs with statistical differences (P < 0.05). To obtain an overall view of differential expression in these miRNAs, Fig. 2B was plotted to show both fold change and statistical P values for each miRNA. As shown, miR-10b, miR-196a, and miR-196b were significantly elevated (>50-fold) in oral cancer cell lines (P < 0.05).

Minimal effect of miR-10b on cell growth and chemo-/radiosensitivity

Because miR-10b consistently showed highest level of overexpression in oral cancer cells in both microarray and RT-qPCR assays, this molecule was selected for further functional investigation. An in vitro loss-of-function analysis was applied, which silenced the miRNAs using antagomir oligonucleotides. Results showed that anti-10b caused substantial levels of inhibition, with approximately 90% reduction compared with the scramble control at day 1 (Supplementary Fig. S2A). The 1-day treatment using 150 μmol/L of anti-miR10b was applied in subsequent cellular studies.

The potential effect of miR-10b on cell growth was examined in 2 oral cancer lines, OECM1 and SAS. Treatment with the specific miR-10b antagomir had no effect on cell growth or colony formation in either cell line (Supplementary Fig. S2B). The potential effect of miR-10b on chemo-/radiosensitivity was also examined. Also, treatment with the specific miR-10b antagomir had no differential effect on cell survival in response to drug or irradiation (Supplementary Fig. S2C).

Positive regulation of miR-10b on cell migration and invasion

Cell migration and invasion were determined using in vitro wound-healing and Matrigel invasion assays in 2 oral

Table 2. Summary of the 23 miRNAs with relative fold changes and P values between 6 oral cancer cell lines and 5 lines of normal keratinocytes, as determined by miRNA array method

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Cancer (C)</th>
<th>Normal (N)</th>
<th>Fold (C/N)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-10b</td>
<td>2.217 (1.68)</td>
<td>0.01 (0.00)</td>
<td>221.7</td>
<td>0.047</td>
</tr>
<tr>
<td>miR-9'</td>
<td>2.175 (0.91)</td>
<td>0.197 (0.30)</td>
<td>11.04</td>
<td>0.009</td>
</tr>
<tr>
<td>miR-196a</td>
<td>4.06 (3.22)</td>
<td>0.236 (0.22)</td>
<td>17.20</td>
<td>0.064</td>
</tr>
<tr>
<td>miR-196b</td>
<td>1.349 (0.38)</td>
<td>0.123 (0.11)</td>
<td>10.96</td>
<td>0.002</td>
</tr>
<tr>
<td>miR-582-5p</td>
<td>1.698 (0.45)</td>
<td>0.416 (0.25)</td>
<td>4.08</td>
<td>0.744E-05</td>
</tr>
<tr>
<td>miR-15b</td>
<td>1.582 (0.42)</td>
<td>0.399 (0.22)</td>
<td>3.96</td>
<td>0.002</td>
</tr>
<tr>
<td>miR-301</td>
<td>1.866 (0.61)</td>
<td>0.498 (0.30)</td>
<td>3.74</td>
<td>0.006</td>
</tr>
<tr>
<td>miR-7</td>
<td>1.645 (0.68)</td>
<td>0.464 (0.37)</td>
<td>3.54</td>
<td>0.021</td>
</tr>
<tr>
<td>miR-148b</td>
<td>1.337 (0.27)</td>
<td>0.450 (0.19)</td>
<td>2.97</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-486</td>
<td>1.525 (0.38)</td>
<td>0.514 (0.24)</td>
<td>2.96</td>
<td>0.003</td>
</tr>
<tr>
<td>miR-93</td>
<td>1.631 (0.43)</td>
<td>0.579 (0.21)</td>
<td>2.82</td>
<td>0.004</td>
</tr>
<tr>
<td>miR-652</td>
<td>1.347 (0.63)</td>
<td>0.488 (0.21)</td>
<td>2.76</td>
<td>0.042</td>
</tr>
<tr>
<td>miR-30a-3p</td>
<td>1.788 (0.53)</td>
<td>0.658 (0.21)</td>
<td>2.72</td>
<td>0.010</td>
</tr>
<tr>
<td>miR-30e-3p</td>
<td>1.521 (0.71)</td>
<td>0.579 (0.31)</td>
<td>2.63</td>
<td>0.041</td>
</tr>
<tr>
<td>miR-128b</td>
<td>1.255 (0.24)</td>
<td>0.484 (0.26)</td>
<td>2.59</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-324-5p</td>
<td>1.281 (0.24)</td>
<td>0.494 (0.27)</td>
<td>2.59</td>
<td>0.002</td>
</tr>
<tr>
<td>miR-25</td>
<td>1.327 (0.26)</td>
<td>0.536 (0.21)</td>
<td>2.47</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-18a</td>
<td>1.529 (0.44)</td>
<td>0.646 (0.17)</td>
<td>2.37</td>
<td>0.014</td>
</tr>
<tr>
<td>miR-128a</td>
<td>1.402 (0.33)</td>
<td>0.597 (0.29)</td>
<td>2.30</td>
<td>0.004</td>
</tr>
<tr>
<td>miR-638</td>
<td>0.661 (0.32)</td>
<td>1.618 (0.75)</td>
<td>0.409</td>
<td>0.051</td>
</tr>
<tr>
<td>miR-503</td>
<td>0.663 (0.32)</td>
<td>1.477 (0.38)</td>
<td>0.449</td>
<td>0.009</td>
</tr>
<tr>
<td>miR-22</td>
<td>0.833 (0.12)</td>
<td>1.720 (0.38)</td>
<td>0.485</td>
<td>0.004</td>
</tr>
<tr>
<td>miR-31</td>
<td>0.677 (0.24)</td>
<td>1.359 (0.12)</td>
<td>0.498</td>
<td>0.046E-04</td>
</tr>
</tbody>
</table>

NOTE: The miRNA expression profile was determined using a 470 miRNA array method (Agilent Technology). Using ANOVA with FDR < 0.1 and greater than 2-fold change of expression as selection criteria, 23 miRNAs were found to be significantly differentially expressed between normal and cancer cells.

Abbreviations: C, oral cancer cell lines; N, normal keratinocytes.
cancer cell lines. For the migration assay, miR-10b antagomir transfectants showed slower migration toward the gap area than the controls (Fig. 3A). At 12 hours, the miR-10b antagomir transfected cells showed 48% and 38% reduction in OECM1 and SAS cells, compared with the controls (Fig. 3B). For the invasion assay, cells were seeded in the upper chamber and allowed cells invading to the outer surface of the Matrigel. As shown, the invaded cells of miR-10b antagomir transfectants were dramatically reduced compared with the controls (Fig. 3C), with reduction to 58% and 52% at 24 hours, respectively, in OECM1 and SAS cells (Fig. 3D). Apparently, suppression of miR-10b inhibited the invasion and migration ability of oral cancer cells.

Examination of potential target gene of miR-10b in oral cancer cells

Several target genes of miR-10b have been reported (20–24), so that we investigated whether the alterations of cell functions by miR-10b in oral cancer cells may through these genes. They are HOXD10, the prometastatic gene, that leads to RhoA/RhoC upregulation (20–22); Tiam1, the Rac-activating protein (23); and KLF4 cancer stem cell–associated gene (24). To clarify whether these molecules were associated with miR-10b, we have examined the expression levels of these potential target genes (HOXD10, RhoA/RhoC, Tiam1, and KLF4) in the cell lines versus normal keratinocytes and in antagomir-expressing cell lines. Results are
Figure 3. Positive regulation of miR-10b on cell migration and invasion. A, effects on cell migration as determined by in vitro wound-healing assay. Cells, either transfected with miR-10b antagonir (Anti-10b) or the scramble oligonucleotides, were seeded in an ibidi culture insert (Applied BioPhysics, Inc.) on the top of a 6-well plate for 8 hours. The culture insert was then detached to form a cell-free gap in a monolayer of cells. After changing to culture medium with 1% fetal calf serum, cell migration toward the gap area was photographed after 6 hours. B, the quantitative results of the in vitro wound-healing cell migration assay at 6 and 12 hours. C, effects on cell invasion as determined by Matrigel invasion assay. Cells, either transfected with miR-10b antagonir or the scramble oligonucleotides, were seeded on the upper wells of the Millicell chamber (Millipore) coated with Matrigel (Becton Dickinson Biosciences). The lower chamber contained complete culture medium, which included 10% FBS to trap invading cells. After incubation at 37°C for 16 hours, the number of cells invading to the outer surfer of the Matrigel was stained and photographed. D, the quantitative results of Matrigel invasion assay for 12 and 24 hours. The number of cells invading through the Matrigel to the lower chamber was determined. Each experiment was carried out in duplicate. SC, scramble.
shown in the Supplementary Fig. S3. Unfortunately, none of these genes showed convincingly increased expression in antagonir-treated cells (Supplementary Fig. S3A and S3B). Furthermore, results of HOXD10 were opposite of what was expected, which was increased in cancer cell lines compared with normal keratinocytes. Therefore, the downstream regulatory pathway of miR-10b leading to cell invasion in oral tissues may not be through these molecules and is awaited to be further investigated.

Elevation of plasma miR-10b in oral pre-cancer and cancer patients, as well as in mice with xenografted tumors

To assess the association of miR-10b in cancer formation, xenografted SAS tumors in mice were established. A total of $1 \times 10^6$ cells were subcutaneously injected into the upper area of the hind limb, and tumors were allowed to form for 6 weeks. Plasma samples were collected before and 6 weeks after tumor injection, and miR-10b expression levels were determined. Results are shown in Fig. 4A. Although varying in size, all the tumors were more than 1,000 mm$^3$ in volume in the 4 xenografted mice, ranging from 1.1 cm$^3$ to 3.1 cm$^3$. Plasma miR-10b was significantly elevated in all mice, after tumor formation, with an average increase of approximately 20-fold (3.54 to 71.63; $P = 0.043$).

To further assess potential clinical applications of miR-10b, its expression was determined in 93 plasma samples, including 36 from normal donors, 7 from patients with precancer, and 54 patients with oral cancer. As shown in Fig. 4B, the level of miR-10b in patients with cancer was significantly higher than in normal subjects, with approximately 4-fold of elevation in average ($P < 0.0001$). The expression level of miR-10b in plasma of patients with precancer was also shown with a striking result that average value of precancer was equal to that of patients with oral cancer, significantly higher than in
normal subjects. However, it showed no significance in different T stage ($P = 0.455$), N stage ($P = 0.450$), and overall stage ($P = 0.417$). Using ROC analysis, this molecule yielded an AUC of 0.932 and 0.967 in oral cancer and precancer samples, respectively (Fig. 4C and D). A logistic regression model with the best prediction rate yielded 94.4% sensitivity and 80.0% specificity in distinguishing patients with oral cancer from the healthy controls. These results indicate that miR-10b has potential as an early detection marker for oral cancer.

Discussion

In this study, we profiled oral cancer–specific patterns of miRNA expression using 6 oral cancer cell lines and 5 lines from oral normal keratinocytes with 470 miRNA array analyses. Microarray data revealed that oral cancer cell lines show a distinct pattern of microRNA expression, compared with normal keratinocytes. After clustering analysis, 23 miRNAs were found to be significantly differentially expressed between the 2 groups (Fig. 1, Table 1). The RT-qPCR method independently examined these 23 miRNAs and found 10 miRNAs consistent with the array results that showed significant differential expression (>2-fold) and low $P$ values ($<0.05$; Fig. 2, Supplementary Table S1); these data suggest that these miRNAs are important in oral cancer formation. Upregulation of miR-15b and miR-128a, and downregulation of miR-31 and miR-503, were concordant with previous reports in head and neck cancer (8, 11, 25, 26). Upregulation of miR-10b, miR-148b, miR-196a, miR-196b, miR-582, and miR-301 and downregulation of miR-503 were newly observed in head and neck cancer. However, these novel miRNAs have been reported to be associated with other cancers. These data support the previously suggested participation of these miRNAs in malignant transformation, as oncomir functions of miR-10b in breast and esophageal cancers (20, 24), miR-196 in colorectal and gastric cancers (27), miR-301 in liver cancer (28), and miR-148b in lung cancer (29).

Although miR-10b has been reported to promote cancer metastasis in several cancers, little is known about this molecule in oral cancer. In agreement with previous reports, we found that silencing miR-10b significantly decreased cellular migratory and invasive ability, suggesting positive regulatory roles for this molecule in oral cancer cells as well (Fig. 3). Several genes have been reported as the potential targets of miR-10b, such as HOXD10, RhoA/RhoC, Tiam1, and KLF4 (20–24). However, we did not find significant associations in any of these molecules with miR-10b expression (Supplementary Fig. S3). Therefore, the downstream regulatory pathway of miR-10b leading to cell invasion in oral tissues is awaited to be further investigated. We further noted that miR-10b has a minimal role in cell growth regulation or the stress response (Supplementary Fig. S2). All these results help elucidate the function of miR-10b in the carcinogenesis of oral cancer.

Recently, several studies showed that miRNAs can be stably expressed in plasma, serum, and other body fluids, indicating a potential for the use of miRNAs as clinical biomarkers (30–32). In our xenografted tumor study, we found that circulating miR-10b dramatically increased (20-fold) in the plasma after tumor development (Fig. 4A), indicating that this molecule may potentially be used as a diagnostic marker. However, the level of miR-10b elevation was not correlated to tumor size. This result may reflect the fact that the miR-10b level was more strongly associated with cell invasion ability and less strongly associated with tumor growth (Fig. 3). It was also supported by a previous report that the administration of miR-10b antagomirs significantly suppressed tumor metastasis but not the growth of the primary tumor (33). Because circulating miR-10b was remarkable increased after xenografted tumor formation, we determined the differential expression of this molecule in patients with oral cancer and precancer and normal volunteers. We found that miR-10b was significantly upregulated in the plasma from patients with oral cancer compared with the controls with high AUC value (0.932, $P < 0.0001$; Fig. 4C). Remarkably, the elevation of miR-10b in patients with precancer plasma was close to that in patients with cancer, suggesting that miR-10b possesses a good predictive ability for early detection of patients with oral cancer (Fig. 4B–D). In conclusion, we have profiled at least 10 miRNAs significantly associated with oral cancer, including miR-10b. Through promoting cell migration and invasion, miR-10b actively participates in cancer formation. Our study in clinical samples suggests that plasma miR-10b may have a high potential as a novel less-invasive biomarker for the early detection of oral cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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