Oncogenic function and early detection potential of miRNA-10b in oral cancer as identified by microRNA profiling

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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.

Journal category: Early detection research  
Running title: oncogenic miR-10b shows early detection potential in oral cancer  
Key words: miR-10b, oral cancer, cell invasion, circulating miRNA, early detection
Abstract

MicroRNA (miRNA) participates in a variety of biological 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 miRNA found considerably differential expressions between 6 oral cancer cell lines and 5 lines of normal oral keratinocytes. In which, 10 miRNAs showed the highest significant difference after independent examination by RT-qPCR. Eight molecules were up-regulated; miR-10b, miR-196a, miR-198b, miR-582-5p, miR-15b, miR-301, miR-148b, and miR-128a; and 2 molecules-miR-503 and miR-31 were down-regulated. The most up-regulated 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-/radio- sensitivity. Furthermore, miR-10b was considerably elevated in the plasma of xenografted tumor mice (20-fold). This up-regulation of miR-10b in plasma was further demonstrated in the patients with oral cancer ($P < 0.0001$, AUC=0.932) and pre-cancer lesions ($P < 0.0001$, AUC=0.967), suggesting miR-10b possesses a high potential to discriminate the patients with normal subjects. In conclusion, we have identified at least 10 miRNAs significantly associated with oral cancer, including most elevated miR-10b. The miR-10b actively participates in cancer formation through 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.
Introduction

Oral cancer is one of the most common cancers worldwide, with an incidence estimated to be over 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 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.

MicroRNAs (miRNAs) are endogenous, small non-coding 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 over 700 human miRNAs annotated in the Sanger miRBase database, and it has been predicted that are more than 1000 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 biological 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 performed 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 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-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 demonstrated its highly diagnostic potential for early detection in 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) and OC3 (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, Taiwan. SAS, OECM1 and OC3 cell lines were kindly distributed from Professors SC Lin, and KW Chang, Yang Ming University, 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, 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) HPV immortalization (Chang Gung Memorial Hospital, Taiwan). All the cell lines established in Taiwan (OECM1, OC3, CGHNC8, CGHNC9, CGHK2, CGHNK4, CGK1, CGK5, CGK6) were derived from oral cancer patients 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
original authenticated using the experiments described before. All the cells used were re-tested on the basis of viability, growth status, morphology and the status of HPV infection (17). All the cells were used within 3 months after resuscitation of the frozen aliquots, with lower than 20 passages in each experiment. For HPV status, all the cell lines were negative for HPV-16 and HPV-18, except SAS and OC3 were weak positive for HPV-18. The normal keratinocyte cells were maintained in KSFM medium (Gibco BRL, Rockville, MD). OECM1 cells were grown in RPMI 1640 medium, and all other cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1X antibiotics-antimycotics (Gibco BRL). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ air.

**Profiling of miRNA expression and data analysis**

Total RNA from cells was isolated with TRizol reagent (Gibco BRL) following the manufacturer’s instructions. The concentration, purity, and amount of total RNA were quantified using the Nano-Drop ND-1000 ultraviolet Spectrophotometer. Agilent’s miRNA microarray system (G4470A, Agilent Technologies Inc., United Kingdom), which contains 470 human miRNAs, was used for expression profile analysis. For each microarray analysis, 1 ug of total RNA was used according to manufacturer’s instructions. Slides were scanned on a microarray scanner (model G2565A, Agilent) with a high dynamic range setting, and the Feature Extraction software (Agilent) was used for data extraction.

GeneSpring GX software (version 7.3.1, Agilent) was used to analyze the expression level of miRNAs from the microarray assay. Data were filtered by Agilent present/absent flags, and the intensity level was set to exclude weak signals. The analysis of variance (ANOVA) with the Benjamini and Hochberg correction for false-positive reduction was used to find differentially expressed miRNAs with a false discovery rate (FDR) < 0.1.
Those miRNAs showing more than a two-fold-change between the cancer and the normal group were selected by an n-fold-change filter tool. Hierarchical cluster analysis was applied to average linkage using Pearson correlation as a measure of similarity between sample groups.

*Reverse transcription quantitative PCR (RT-qPCR) analysis for miRNA validation*

Total RNA was isolated from cells with TRizol reagent (Gibco BRL, Rockville, MD). Reverse transcription was performed as previously described (18), except for the use of miRNA specific stem-loop RT primers. TaqMan® miRNA assays kits (ABI, Forest City, CA) were used to examine specific miRNA expression by RT-qPCR, 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 (ΔΔCt) was used to determine miRNA expression.

*Transfection of miRNA antagomir oligonucleotide*

Cells were seeded for transfection at a density of 5 x 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 μM) of miR-10b antagomir or scramble oligonucleotides, by using Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA) in OPTI-MEM medium (Invitrogen, Carlsbad, CA) for 16 hours; then, the medium was replaced with fresh complete medium. The nucleotide sequence of miR-10b antagomir was 5’-CAC AAA TTC GGT TCT ACA GGG TA-3’. The scramble oligonucleotides contained the same nucleotides as the miR-10b antagomir but in random sequence, 5’ CTG TTC GCA CAG CTT GGT TAA AA -3’.

*Colony formation assay*
To determine colony-formation ability, cells were transfected with either antagomir or scramble oligonucleotides. A total of 1000 cells was 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 antagomir or scramble oligonucleotides, 70 µl of 5 × 10⁵ cell/ml transfectants were seeded in an ibidi® culture insert (Applied BioPhysics, Inc. NY) on top of a 6-well plate. After 8 hr 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% FCS, the cell migration status toward the gap area was photographed every 6 hr.

**Matrigel invasion assay**

The cell invasion assay was performed 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 h at 37°C. After transfection with either antagomir or scramble oligonucleotides, cells were seeded into the upper chamber at a density of 1 × 10⁵ cells/well in 0.2 ml 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 were counted and compared to the number of cells that passed through the membrane in the control chambers. The invaded cells on lower side of membrane were fixed, stained by crystal violet and photographed.
Determination of chemo- or radio-sensitivity

Cells, either transfected with antagonim or scramble oligonucleotides, were seeded at 1x10^5 cells/well in 6-well plates for 8 hr. For determination of chemosensitivity, cells were treated with various doses (0 to 80 μg/ml) of cisplatin and continuously cultured for 2 days. The number of surviving cells was counted and compared to the number of surviving untreated cells. For determination of radiosensitivity, the 1000 antagonim 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 to 6 Gy) and continuously cultured for 7 days, followed by 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 oral cancer patients in the week prior to receive operation and/or chemo-radiotherapy. The characteristics of these oral cancer patients were summarized in the Table 1. These included 51 (94%) males and 3 (6%) females, with a mean age 52.0 years old. The EDTA-plasma samples from 36 age- and sex-matched normal individuals and 7 oral precancer leukoplaikia patients were also obtained to compare. The miRNA of each specimen was purified from 200 μl plasma using the miRNeasy® mini kit (Qiagen Inc., Valencia, CA) and dissolved in 20 μl of RNase-free water. MiRNA levels 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 x width x 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-squared t-test was used to determine whether miRNA levels were different between the two groups of samples. A P-value of $< 0.05$ was considered statistically significant. To evaluate whether RT-qPCR data could separate differences between normal individuals and cancer patients, receiver operating characteristic (ROC) analysis was performed, and the area under curve (AUC) was used to measure the level of separation between the two groups.

**Results**

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

A miRNA microarray (Agilent Technology, USA) 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 Figure 1A. After normalization and filtering to exclude weak signals, 190 miRNAs were selected for clustering analysis. Unsupervised hierarchical clustering analysis and the analysis of variance (ANOVA) was used to find differentially
expressed miRNAs. With a FDR<0.1 and more than a 2-fold change between the cancerous and the normal group were selected, 23 miRNAs were found with 19 up-regulated and 4 down-regulated in the cancer samples (Figure 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 over-expression (> 10-fold). These results suggest that these 23 miRNAs may play important roles in oral carcinogenesis.

**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 were summarized in the Supplement Table 1. Figure 2A shows representative miRNAs that were significantly differentially expressed between cancer and normal cells (also see in Supplement Figure 1). There were 20 miRNAs confirmed to have over 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, Figure 2B was plotted to show both fold change and statistical \( P \)-value 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-/radio- sensitivity**

Because miR-10b consistently showed highest level of over-expression 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 anti-10b caused
substantial levels of inhibition, with approximately 90% reduction compared to the scramble control at day 1 (Supplement Figure 2A). The 1-day treatment using 150 µM of anti-miR10b was applied in subsequent cellular studies.

The potential effect of miR-10b on cell growth was examined in two 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 (Supplement Figure 2B). The potential effect of miR-10b on chemo-/radio-sensitivity was also examined. Also, treatment with the specific miR-10b antagomir had no differential effect on cell survival in response to drug or irradiation (Supplement Figure 2C).

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 two oral cancer cell lines. For the migration assay, miR-10b antagomir transfectants showed slower migration toward the gap area as compared to the controls (Figure 3A). At 12 hours, the miR-10b antagomir transfected cells showed 48% and 38% reduction in OECM1 and SAS cells, compared to the controls (Figure 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 to the controls (Figure 3C), with reduction to 58% and 52% at 24 hours respectively in OECM1 and SAS cells (Figure 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 pro-metastatic gene that leads to RhoA/RhoC up-regulation (20-22); Tiam1, the Rac activating protein (23); and the 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 verse normal keratinocytes and in antagonir expressing cell lines. Results were showed in the Supplement Figure 3. Unfortunately, none of these genes showed convincingly increased in antagonir-treated cells (Supplement Figure 3A-B). Furthermore, results of HOXD10 was opposite of 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 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 mice with xenografted tumors_

To assess the association of miR-10b in cancer formation, xenografted SAS tumors in mice were established. A total of 1x10^6 cells were subcutaneously injected into the upper area of the hind limb, and tumors were allowed to form 6 weeks. Plasma samples were collected before and 6 weeks after tumor injection, and miR-10b expression levels were determined. Results are shown in Figure 4A. Although varying in size, the tumors were all over 1000 mm³ in the four xenografted mice, ranging from 1.1 cm³ to 3.1 cm³. Plasma miR-10b was significantly elevated in all mice, post-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 pre-cancer patients and 54 from oral cancer patients. As shown in Figure 4B, the level of miR-10b in cancer patients 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 pre-cancer patients was also shown striking result, that average value of pre-cancer was as equal as which of oral cancer patients, significantly higher than in normal subjects. However, it was shown no significance in different T stage ($P=0.455$), N stage ($P=0.450$) and overall stage ($P=0.417$). Using receiver operational curve (ROC) analysis, this molecule yielded an area under curve (AUC) of 0.932 and 0.967 in oral cancer and pre-cancer samples respectively (Figure 4C-4D). A logistic regression model with the best prediction rate yielded 94.4% sensitivity and 80.0% specificity in distinguishing oral cancer patients 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 two groups (Figure 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 ($<0.05$) (Figure 2, Supplement Table 1); these data suggest these miRNAs are important in oral cancer formation. Up-regulation of miR-15b and miR-128a, and down-regulation of miR-31 and miR-503, were concordant with previous reports in head-neck cancer (8, 11, 25, 26). Up-regulation of miR-10b, miR-148b, miR-196a, miR-196b, miR-582, and miR-301 and down-regulation of miR-503 were newly observed in head-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-196s 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 (Figure 3). Several genes have been reported as the potential targets of miR-10b, as HOXD10, RhoA/RhoC, Tiam1 and KLF4 (20-24). However, we did not found significant associations in any of these molecules with miR-10b expression (Supplement Figure 3). 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 (Supplement Figure 2). 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 (Figure 4A), indicating 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 (Figure 3). It was also supported by a previous report that the administration of miR-10b antagonirs significantly suppressed tumor metastasis but not the growth of the primary tumor (33). Since circulating miR-10b was remarkable increased after xenografted tumor formation, we determined the differential expression of this molecule in oral cancer, pre-cancer patients and normal volunteers. We
found that miR-10b was significantly up-regulated in the plasma from patients with oral cancer compared to the controls with high AUC value (0.932, P < 0.0001) (Figure 4C). Remarkably, the elevation of miR-10b in pre-cancer patients’ plasma was close to which in cancer patients, suggesting that miR-10b possesses a good predictive ability for early detection of oral cancer patients (Figure 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.

Acknowledgement

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References

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16


Table 1. Clinical characteristics of patients recruited in this study.

<table>
<thead>
<tr>
<th>Item</th>
<th>Cancer</th>
<th>Precancer</th>
<th>Normal</th>
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</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>51 (94%)</td>
<td>7 (100%)</td>
<td>31 (86%)</td>
</tr>
<tr>
<td>Female</td>
<td>3 (6%)</td>
<td>0 (0%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>52.0 ± 12.8</td>
<td>51.9 ± 10.2</td>
<td>50.5 ± 12.6</td>
</tr>
<tr>
<td>&lt; 40 years old</td>
<td>11 (20%)</td>
<td>1 (14%)</td>
<td>6 (17%)</td>
</tr>
<tr>
<td>41-50 years old</td>
<td>15 (28%)</td>
<td>2 (29%)</td>
<td>11 (31%)</td>
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<tr>
<td>51-60 years old</td>
<td>14 (26%)</td>
<td>2 (29%)</td>
<td>12 (34%)</td>
</tr>
<tr>
<td>≥ 61 years old</td>
<td>14 (26%)</td>
<td>2 (29%)</td>
<td>6 (17%)</td>
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<tr>
<td><strong>Habits</strong></td>
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<tr>
<td>Alcohol drinking</td>
<td>39 (73%)</td>
<td>2 (29%)</td>
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<tr>
<td>Betel quid chewing</td>
<td>40 (74%)</td>
<td>2 (29%)</td>
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<tr>
<td>Cigarette smoking</td>
<td>42 (78%)</td>
<td>6 (86%)</td>
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<tr>
<td><strong>T stage</strong></td>
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<tr>
<td>T1-T2</td>
<td>31 (57%)</td>
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<tr>
<td>T3-T4</td>
<td>23 (43%)</td>
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<tr>
<td><strong>N stage</strong></td>
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<tr>
<td>N = 0</td>
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<tr>
<td>N &gt; 0</td>
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<td><strong>Overall stage</strong></td>
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<tr>
<td>I-II</td>
<td>20 (37%)</td>
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</tr>
<tr>
<td>III-IV</td>
<td>34 (63%)</td>
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<td><strong>Pathological diagnosis</strong></td>
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<tr>
<td>Squamous hyperplasia</td>
<td></td>
<td>4 (57%)</td>
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<tr>
<td>Verrucous hyperplasia</td>
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<td>1 (14%)</td>
<td></td>
</tr>
<tr>
<td>Hyperkeratosis</td>
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<td>2 (29%)</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>54 (100%)</td>
<td>7 (100%)</td>
<td>36 (100%)</td>
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</table>
Table 2. Summary of the 23 miRNAs with relative fold changes and P-values between 6 oral cancer cell lines (C) and 5 lines of normal keratinocytes (N), as determined by miRNA array method. The miRNA expression profile was determined using a 470-miRNA array method (Agilent Technology). The analysis of variance (ANOVA) with FDR<0.1 and greater than two-fold change of expression as selection criteria, 23 miRNAs were found to be significantly differentially expressed between normal and cancer cells.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Cancer (C) Mean (SD)</th>
<th>Normal (N) Mean (SD)</th>
<th>Fold (C/N)</th>
<th>P-value</th>
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</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>
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<td>0.009</td>
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<td>miR-196a</td>
<td>4.06 (3.22)</td>
<td>0.236 (0.22)</td>
<td>17.20</td>
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<td>miR-196b</td>
<td>1.349 (0.38)</td>
<td>0.123 (0.11)</td>
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<td>miR-582-5p</td>
<td>1.698 (0.45)</td>
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<td>miR-15b</td>
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<td>miR-301</td>
<td>1.866 (0.61)</td>
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<td>miR-7</td>
<td>1.645 (0.68)</td>
<td>0.464 (0.37)</td>
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<td>miR-148b</td>
<td>1.337 (0.27)</td>
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<td>miR-486</td>
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<td>miR-93</td>
<td>1.631 (0.43)</td>
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<td>miR-652</td>
<td>1.347 (0.63)</td>
<td>0.488 (0.21)</td>
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<td>miR-30a-3p</td>
<td>1.788 (0.53)</td>
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<td>miR-30e-3p</td>
<td>1.521 (0.71)</td>
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<td>miR-128b</td>
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<td>miR-324-5p</td>
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<td>miR-25</td>
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<td>miR-638</td>
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<td>1.618 (0.75)</td>
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<td>miR-503</td>
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<td>1.477 (0.38)</td>
<td>0.449</td>
<td>0.009</td>
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<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>
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<td>miR-31</td>
<td>0.677 (0.24)</td>
<td>1.359 (0.12)</td>
<td>0.498</td>
<td>2.96E-04</td>
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Figure Legends

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. The analysis of variance (ANOVA) with FDR<0.1 and a greater than two-fold change of expression as selection criteria, 23 miRNAs were found to have significantly different expression levels between normal and cancerous cells.

Figure 2. The expression levels of 23 miRNAs in 6 oral cancer cell lines and 5 lines of normal oral keratinocytes, as determined by RT-qPCR. For each miRNA, the expression levels as recorded by threshold cycle numbers (Ct), were normalized against an internal control (U6 RNA), and the comparative threshold cycle method (ΔCt) was used to determine relative miRNA expression. (A) The relative expression of 4 representative miRNAs is shown. The samples are indicated at the bottom of each figure, six samples of cancer cells (left) and five samples of normal cells (right). (B) The overall view of differential expression between cancerous and normal samples of 23 miRNAs, with the average fold change (X-axis) and statistical P-value (Y-axis) for each miRNA.

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 antagomir or the scramble oligonucleotides, were seeded in an ibidi® culture insert (Applied BioPhysics, Inc. NY) on the top of a 6-well plate for 8 hr. The culture insert was then detached to form a cell-free gap in a monolayer of cells. After changing to culture medium with 1% FCS, cell migration toward the gap area was photographed 6 hr. (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 antagomir 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 surface 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 performed in duplicate.

**Figure 4.** Elevation of plasma miR-10b in oral cancer patients and xenografted tumor mice (A) Determination of circulating miR-10b in the plasma of mice before and after tumor xenografting. A total of 1 x 10^6 oral cancer cells (SAS) were injected subcutaneously in the upper hind limb area of the mice (n=5). After 6 weeks, the sizes of tumor were determined as indicated by the line. Plasma samples from the mice were collected before tumor cell injection (n=4, due to insufficient plasma sample could be obtained) and post-tumor formation (n=5). The circulating miRNA levels of the mice were determined by RT-qPCR, as indicated by the bar and showing the values on the top of each bar. (B) The scatter dot plot shows the relative levels of circulating miR-10b in the plasma from 32 normal individuals, 7 pre-cancer patients and 54 oral cancer patients, as measured by RT-qPCR. The receiver operating characteristic (ROC) analyses for the level of circulating miR-10b across samples of the normal individuals and cancer patients (C) or normal individuals and pre-cancer patients (D). The area under curve (AUC) was used to measure the level of separation between two groups.
Figure 2A

- miR-10b: P = 0.019
- miR-196a: P = 0.035
- miR-15b: P = 0.002
- miR-196b: P = 0.002
Figure 3A-B

(A) OECM1

Scramble Anti-10b

0 hr

6 hr

12 hr

SAS

Scramble Anti-10b

(B) OECM1

Relative level (fold)

SC Anti-10b

0 0.5 1 1.5

6 12

SAS

Relative level (fold)

SC Anti-10b

0 0.5 1 1.5

6 12
Figure 3C-D

(C) OECM1

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(D) OECM1

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<td>12</td>
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</tr>
<tr>
<td>24</td>
<td>2.3</td>
<td>3.2</td>
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</tbody>
</table>
Figure 4A

(A)

Tumor size (mm²)

Pre-xenografted  | Post-xenografted
--- | ---
Mean of miR-10b = 3.5 | Mean of miR-10b = 71.6

- miR-10b
- Tumor size

0 | 1 | 3 | 8 | 2 | 21 | 43 | 148 | 110 | 37

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Oncogenic function and early detection potential of miRNA-10b in oral cancer as identified by microRNA profiling

Ya-Ching Lu, Yin-Ju Chen, Hung-Ming Wang, et al.

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