Profiling of microRNA in oral cancer cells identifying miR-10b functions in oncogenesis and up-regulates in plasma of oral cancer patients

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Abstract

Oral cancer is the 6th leading cancer, and is still increasing with years. Since this cancer usually occurs in the middle age male, at the high peak of life responsibility, it has tremendous impact of family and society. MicorRNAs (miRNAs) are endogenous, non-coding small RNA, which consist of 21-23 nucleotides. They serve widespread functions as regulatory molecules in post-transcriptional gene silencing for the regulation of cellular bio-functions. And the dysregulation of miRNAs has been implicated in malignancy. Agilent system was used to perform miRNA profiling in 6 cancer cell lines and 5 lines of normal keratinocytes for identification of differently expressed miRNAs. There were 23 miRNAs selected as candidates which were shown more than 2-fold change with the FDR<0.1. Among these miRNAs, 19 of them were elevated and 4 were decreased in oral cancer cell lines. miR-10b was validated by qPCR analysis showed significantly up-regulated in cancer cells. Furthermore, the biological function of miR-10b was investigated after alteration of miR-10b expression. Reduction of miR-10b by antisense oligonucleotide further resulted in retard of cell migratory and invasive ability, however, no significant alterations on cell growth or chemo/radio-sensitivity. In analyzing miR-10b expression in 24 normal and 29 plasma samples from oral cancer patients, miR-10b was up-regulated 3.3 fold in the cancer patients (P<0.0001). Taken together, miR-10b plays a role in the carcinogenesis through promoting cancer cell migratory and invasive function, and plasma miR-10b may serve as a biomarker for oral cancer patients.

Key words: microRNA, oral cancer, miR-10b
Introduction

Oral cancer is one of the most frequent cancers worldwide, with an estimated 500,000 or more new cases diagnosed annually [1]. The disease is more prevalent among males than females [1]. Epidemiologic studies have shown a strong association between oral and environmental carcinogens, especially the use of tobacco, alcohol, and betel quid [2-5]. The 5-year mortality rate for oral 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 the quality of life. The reported 5-year disease-specific survival rates in Stage II, III, and IV oral cancer patients are 72-90%, 39-85%, 27-70%, and 12-50 %, respectively [4-6]. The genetic alterations leading to carcinogenesis of head-neck cancer are remained to be identified.

MicroRNAs (miRNA) have been found associated with several types of cancers. miRNA are endogenous, small non-coding RNAs (20-22 nucleotides) that negatively regulate gene expression at the translational level by base pairing to the 3’-untranslated region of target mRNAs [7,8]. The systematic cloning of small RNAs from diverse organisms from plants to humans revealed an increasing number of small RNAs that has been conserved during evolution. There are now more than 500 human miRNAs annotated in the miRNA registry [8,9], and has been predicted the miRNA number to over 1000 [9]. It is estimated that approximately 30% of the human genes are regulated by miRNA, and each miRNA is supposed to target several hundreds of transcripts [10], making miRNAs one of the biggest family of gene regulators.

Recently, large scale of miRNA screening have been performed in several cancers and show unique expression profiles associated with clinical features, including several solid tumors [11], lung cancer [12], colorectal cancer [13], breast cancer [14], hepatocellular carcinoma [15], cervical carcinoma [16], ovarian carcinoma [17] and chromic lymphocytic
leukemia [18]. Several miRNAs have been identified associated with cancer. For examples, miR-143 and miR-145 have been found down-regulated in colorectal cancer [19], and let-7a in lung cancer [20]. Up-regulations of miR-200a or miR-200c correlated with poor survival of ovarian cancer [17]. Several targets of these miRNAs are experimental proved as oncogenes or tumor suppressors, such as miR-106 target to Rb gene and miR-20 to TGF-b receptor II [11].

Since miRNAs serve functions as regulatory molecules in post-transcriptional gene silencing for the regulation of cellular proliferation, differentiation, and apoptosis, which have great impacts on the malignant transformation, the expression of miRNA profile is closely associated with cancer. The aim of this study is to profiling and characterization of the miRNA signature in head-neck cancer. This knowledge will provide basic information for the potential applications of these molecules in the diagnosis and treatment of head neck cancer.
Material and method

Cells and Cell lines

Total of 6 oral cancer lines (OECM1, SAS, SCC25, OC3, CGHNC8 and CGHNC9) and 5 normal keratinocytes were used (CGHNK2, CGHNK4, K1, K5, K6) in miRNAs profiling. CGHNK2 and CGHNK4 were HPV-immortalized lines of normal oral keratinocytes. Yo, Su and Ts were normal mucosa cells from healthy individuals. The normal keratinocyte cells were maintained in KSFM medium (Life Technologies, Inc., GibcoBRL, Rockville, MD). Other cancer cell lines were grown in 100% DMEM/RPMI 1640 medium with 10% fetal bovine serum (Life Technologies, Inc.). All cells will be cultured at 37°C in a humidified atmosphere of 5% CO₂ air.

RNA extraction

Total RNA from cells were isolated with TRizol reagent (Gibco BRL) following the manufacturer’s instructions. The concentrations, purity, and amounts of total RNA were quantified using Nano-Drop ND-1000 ultraviolet Spectrophotometer.

miRNA expression profiling

Agilent’s miRNA microarray system (Agilent Technologies Inc., United Kingdom) was used for expression profiling analysis. For each microarray analysis, 1 ug of total RNA was used according to manufacture’s instruction. Briefly, sample RNAs were dephosphorylated with 11.2 units calf intestine alkaline phosphatase (GE Healthcare Life Sciences, Sweden) for 30 min at 37°C. The reaction was terminated at 100°C for 5 min and immediately cooled to 0°C. 5 μl of DMSO were then added and heated to 100°C for 5 min and immediately cooled to 0°C. Ligase buffer and BSA were added and ligation was performed with 50 uM pCp-Cy3 and 15 units T4 RNA ligase (GE Healthcare Life Sciences, Sweden) in 28 ul at 16°C for 2 h. The labeled miRNAs were desalted with MicroBioSpin6 columns (BioRad, USA). 2X hybridization buffer and blocking reagent (Agilent Technologies, USA) were added to the labeled mixture to a final volume of 45 ul. The mixture was heated for 5 min at 100°C and
immediately cooled to 0°C. Each 45 ul sample was hybridized onto an Agilent human miRNA Microarray v1 (Agilent Technologies, USA) at 55°C for 20 h. After hybridization, slides were washed 5 min in Gene Expression Wash Buffer 1, then for 5 min in Gene Expression Wash Buffer 2, both at room temperature. Slides were scanned on an Agilent microarray scanner (Agilent Technologies, model G2565A) with 5 um scan resolution and the eXtended Dynamic range feature (XDR Hi 100%, XDR Low 10%). Feature Extraction software (version 9.5.3, Agilent Technologies) was used for data extraction.

miRNA array data analysis

GeneSpring GX software (version 7.3.1, Agilent Technologies) was used to analyze the expression levels of miRNAs from microarray assay. Data were filtered by Agilent present/absent flags and intensity level to exclude weak signal. Analysis of variance (ANOVA) with the Benjamini and Hochberg correction for false-positive reduction was used to find differentially expressed miRNAs with FDR<0.1. Those miRNAs showing more than 2-fold-change between cancer and normal group were selected by n-fold-change filter tool. Hierarchical cluster analysis was also applied to average linkage by using Pearson correlation as a measure of similarity between sample groups.

Real-time quantitative reverse transcription –PCR analysis for miRNA validation.

The reverse transcription reaction was performed by incubation of a reaction mixture containing 300 ng total RNA, 3 ul miRNA specific stem-loop RT primer (TaqMan® miRNA assays kit, ABI, Forest City, CA), 4 units of reverse transcriptase (AMV, HT Biotech Ltd, UK), 10 units of RNase inhibitor (CalBiochem, CA, USA), and 25 mM dNTP in a total of 30 ul reaction buffer at 37°C for 30 min. miRNA qPCR was performed by the TaqMan® miRNA assays kit (ABI, Forest City, CA). 1ul miRNA specific probe mixture added with 3 ul RT reaction product, 6 ul 2d-H2O and 10 ul iQ supermix (Bio-Rad, Hercules, CA). Real-time PCR were performed on Bio-Rad MiniOpticon detection system. The real-time PCR results, recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6
RNA), and then expressed as fold expression.

**Antisense oligonucleotide transfection**

OECM1/SAS cells were transfected with various concentrations (75 uM~600 uM) of antisense or the scramble control oligonucleotides against miR-10b, miR-196a, miR196b (as indicated in each line of Fig 5) by lipofectamine reagent. After 24 and 48 hours, cells were harvested for total RNA collection to examine the expression level of miR-10b, by RT-PCR analysis.

**Colony formation assay**

Cells, either transfected with antisense or scramble oligonucleotides, were determined of altering cell ability of colony formation. 1000 cells will be seeded in 6-well plate and allow to growth for up to 7 days without moving. After fixation by 70% ethanol, it was stained with 0.5% crystal violet, and then the cell colonies were counted.

**Matrigel invasion assay**

The invasive ability was determined through culturing the cells in a polycarbonate membrane coated with Matrigel in a Transwell invasion chamber. Cells, transfected with with antisense or scramble oligonucleotides were seeded at a density of 1 x 10^5 per well in 0.2 ml medium at the Matrigel (BD Biosciences) -coated membrane containing invasion chambers (Millipore). Cells were incubated at 37°C for 24 hours. The numbers of cells that invaded through the Matrigel -coated membranes were calculated by comparison with the cells passed through the membranes in the control chambers.
Results and Discussion

Differential miRNA expression profiles between oral cancer cells and keratinocyte, identifying 23 significant miRNAs and miR-10b is the most differently expressed one.

To identify miRNAs differentially expressed in various oral cancer cells and normal keratinocyte, we used a custom microarray (Agilent Technology, USA) which contained 470 human miRNA from the Registry. After normalization and filtering to exclude the weak signal, 192 miRNAs were selected for clustering analysis as shown in the Figure 1. The unsupervised hierarchical clustering analysis was used to classify the samples without using any information on the identity of the samples. As shown, two major classes can be classified based on the similarities in miRNA expression pattern: the cancer cell lines, and normal keratinocytes. Among the 192 miRNAs, there were 23 miRNAs which are significant differentially expressed between oral cancer group and normal group, with 19 up-regulated and 4 down-regulated, as shown in the Figure 2B. The expression trends of these miRNAs were homogenously across almost all samples in the same group. In order to validate the microarray data, real-time quantitative PCR of differently expressed miRNAs were performed independently. The miRNA threshold cycle normalized with internal control- U6 RNA, shown as fold expression. The p-value of miR-10b expression in cancer and normal group was 0.019 (figure 3).

miR-10b promotes oral cancer cells migration/invasion without affecting cell proliferation and have minima effect on oral cancer cell chemo-/radio- sensitivity

The biological functions of these three miRNAs were examined in the potential roles on cell growth, migration, invasion, and the response to radiation and chemotherapy drug. First, the cellular effects of anti-miR-10b were determined. It was observed that treatment with the specific ASO has minimum effect on the cell growth or colony formation (Figure 3A, 3B). These results indicated that miR-10b has no significant function on cell growth regulation.

Cell migration and invasion were analyzed by in vitro wound healing assays and
Matrigel invasion assay. For the migration assay, in both cell lines, the anti-miR-10b transfectants showed a slower migration toward to the gap area compared to controls (Figure 4A). At the 12 hours, the gap area was almost completely covered by the control cells, while the ASO transfected cells were still moving toward into the area. In matrigel invasion assay, the anti-miR-10b transfected cells were seeded in the upper chamber of Matrigel-coated Millicells®. The number of cells invading the lower chamber was determined after 24 hours. As shown in the Figure 4B, significant reduction of anti-miR-10b transfected cells were found invaded to the lower chamber compared to the controls. The invading cells were reduced to approximately 49% and 31% in OECM1 and SAS cells. Apparently, suppressions of miR-10b inhibit the invasion and migration abilities on oral cancer cells.

In order to investigate the cellular effects of miR-10b in chemo- and radio-sensitivity, cell survival fraction were determined after irradiation and cisplatin treatment in miR-10b suppression cell. As shown in the Figure 5A, inhibition of miR-10b by specific ASO has minimum effect on the cytotoxicity to cisplatin treatment. Radiation exposure after reduction of miR-10b expression was shown minimum effect on colony formation (Figure 5B). It was suggested that miR-10b had no significant function on radio-sensitivity of head-neck cancer cells.

miR-10b increase in mice plasma after xenograft and miR-10b elevation in plasma of oral cancer patients

In order to determine whether miR-10b could be secreted out of the tumor cell and further detected in plasma sample, the SAS cell was subcutaneously injected into mice. The cell number was 1*10⁶ per site injected for tumor formation on mice. Post-injection of SAS cell for 6 weeks, tumors are obvious formation at the mean volume approximately 3000 mm³. Plasma was collected before cancer cells injection and post tumor formation. The expression level of miR-10 in mice plasma was detected by RT-qPCR. As shown in figure 6, the miR-10b up-regulated to around 20 fold in tumor burden mice plasma than in pre-injected plasma. The
miR-10b was significantly increase after tumor formation with p-value was 0.043.

Furthermore, MiR-10b expression levels were determined in 93 plasma samples including 32 from normal donor and 61 from oral cancer patients. As shown in figure 5, average value of normal and tumor samples are 0.75 and 2.1, respectively. The miR-10b elevated in plasma of oral-cancer patient with 2.8 fold change. Furthermore, receiver operating curve analysis was done to determine which value showed the greater predictive power (figure 7B). The area under curve (AUC) is 0.87 as good predictive ability. Set the cutoff value at 0.75 showed the 90.16% sensitivity and 68.75% specificity.

**Conclusion and Discussion**

The present studies revealed a number of microRNAs to be aberrantly expressed in head-neck cancer tumors. MiR-21 was reported as a putative oncogenic miRNA in head-neck cancer [22]. Similarly, miR-103/107 were also determined to be up-regulated in head and neck cancer cell line [23]. For potential head-neck cancer biomarkers discovery, the expression ratio of miR-221: miR375 showed a high sensitivity and specificity [24]. However, epidemiologic studies have shown a wide variation range of incidence between worldwide areas [25]. We establish primary tumor and normal cell lines locally for identification of unique miRNAs in head-neck cancer Asia. After miRNAs profiling, miR-10b was identified and confirmed as the most significant miRNA in head-neck cancer. Indeed, our finding was far different from others suggesting that mechanism of head-neck carcinogenesis is different from other region. In this study, we demonstrate that miR-10b positively regulate oral cell migration and invasion without affecting cell growth. As previous study in breast cancer, miR-10b had been reported as an initiator for tumor invasion and metastasis [26]. Due to miRNA expression profiles have been shown to have signature related to tumor classification, diagnosis and disease progression, circulating miRNAs were detected for clinical utility. After subcutaneous injection of SAS cell into mice, the miR-10b was significantly increased in the
plasma, suggesting the bio-marker potential for this miRNA be applied to oral cancer
detection. It had been demonstrated that miR-184 significantly higher in tongue squamous
cell carcinoma patients than in normal [43]. Our data showed significant up-regulation of
miR-10b in oral-cancer plasma compared to the control serum, suggesting miR-10b
potentially serve as a biomarker for oral cancer.
References


Figure 1. (A) Hierarchical clustering analysis of miRNAs between cancer cell lines and normal keratinocytes derived from head-neck tissues. Total of 11 samples were analyzed with a 470 miRNA based microarray assay (Agilent Technology). After filtering out the weak signals in the samples, 190 miRNAs were selected. These miRNAs in 11 samples were subjected to hierarchical clustering analysis. Though each sample showed a distinct expression profile, two groups of samples can be classified. (B) List of 23 miRNAs showing significantly differential expressed between ORAL CANCER cells and normal keratinocytes with FDR less than 0.1 and more than 2-fold change. 19 miRNAs were over-expressed and 4 miRNAs were down-regulated in head-neck cancer samples.
Figure 2. Validation of differential expressions of miR-10b between cancer cells and normal keratinocytes by real-time qPCR analysis.
Figure 3. (A) Cell growth rate were determine after transfected with anti-miR-10b ASO or the scramble oligonucleotides, $1 \times 10^5$ cells were seeded in a 6 well plate and then cultured for up to 3 days. Cell numbers were determined every 24 hours. Experiments were performed in duplicate. (B) After transfection with anti-miR-10b ASO or the scramble oligonucleotides, 1000 cells were seeded in a 6-well plate and incubated for 10 days to allow colony formation. Cell colonies were visualized by 5% crystal violet staining. The relative number of colonies was counted for qualification. Experiments were performed in duplicate.
Figure 4. (A) Effects on cell migration were determined by *in vitro* wound healing assay. After transfection with anti-miR-10b ASO or the scramble oligonucleotides, 70 ul of $6 \times 10^5$ cell/ml transfectants were seeded per well in a Culture-Insert and incubated for 8 hours to allow monolayer cell formation. After removing the Culture-Insert and then incubated cells in the presence of 1% FBS culture medium for up to 12 hours. Cell migration toward the gap was observed and photographed. (B) Effects on cell invasion were determined by Matrigel invasion assay. A total of $1 \times 10^5$ cells transfected with anti-miR-10b ASO or the scramble oligonucleotides were seeded 24 well plate upper well, a Millicell invasion chamber coated with Matrigel, and incubated at 37°C for 24 hours. The numbers of cells invading through the Matrigel to the lower chamber were determined. Each experiment was performed in duplicate.
Figure 5. (A) For chemo-drug sensitivity experiments, $2 \times 10^5$ cells were seeded in a 6 well plate after transfected with anti-miR-10b ASO or the scramble oligonucleotides. After seeding the cell for 8 hours, cells were treated with various doses (0 to 80 ug/ml) of cisplatin and continuously cultured for 2 days. The cell numbers were determined, and compared to the untreated controls. Each experiment was performed in duplicate. (B) In each radiation exposure treatment, 1500 cells were seeded in a 30 mm dish after transfected with anti-miR-10b ASO or the scramble oligonucleotides. Cells were treated with various doses (0 to 6 Gy) of $\gamma$-irradiation and continuously cultured for 14 days to allow colony formation. Cell colonies were visualized by 5% crystal violet staining. The number of colonies was counted for quantification and compared to the control. Each experiment was performed in duplicate.

(A)

(B)
Figure 6. Determination of miR-10b level in mice plasma which collected from pre-xenograft of SAS cell mice and tumor burden mice. $1 \times 10^6$ SAS cell was subcutaneously injected into mice for 6 weeks. Plasma was collected before tumor cell injection and post tumor formation and was detected the expression level of miR-10b by RT-qPCR.
Figure 7. (A) Determination of miR-10b expression in plasma from normal and oral-cancer patients was shown. Total 93 samples containing 32 normal and 61 tumor plasmas were detected for miR-10b. It was shown 2.8-fold higher in tumor than in normal samples with p-value less than 0.0001. (B) Receiver operating curve analysis of miR-10b expression level was performed for differentiation of normal and oral cancer plasma. The area under ROC curve is 0.87.