

MicroRNA profiling in Head and Neck Cancer

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Abstract

Endogenous small non-coding RNA molecules, such as microRNAs (miRNAs) are thought to play an important role in human carcinogenesis. These 21-23 nucleotides of miRNAs will silence gene expressions through binding to the 3’UTR of their target mRNAs. Previous evidence with differential expression of microRNA in cancer and normal samples suggests that they are involved in the development of cancer. To identify the miRNA expression specific for head-neck cancer (HNC), the miRNA profiling of 6 cancer cell lines, 2 immortalized normal cell lines and 3 lines of primary normal cell were performed by miRNA microarray. Total of 470 human miRNA were screened for the differential expressions between cancer and normal cell lines. There were 23 miRNAs expressed significantly between two groups (P < 0.05), with 19 elevated and 4 decreased in HNC cell lines. Of which, 9 miRNAs were further validated using RT-qPCR analysis, including miR-10b. The biological function of miR-10b was investigated. Suppression of miR-10b expression by anti-sense oligonucleotide led to repression of cell migration and invasion phenotype. However, there is no significant effect on cell growth, apoptosis or chemo/radio-sensitivity. In conclusion, we have identified at least 9 miRNAs which associated with HNC. miR-10b plays role in the carcinogenesis through promoting cancer cell migrating and invasive, while no effect on cell viability or stress responses.

Key words: microRNA, Head-neck cancer, miR-10b
Head-neck cancer (HNC) 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 HNC and environmental carcinogens, especially the use of tobacco, alcohol, and betel quid [2-5]. The 5-year mortality rate for HNC 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 µg 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 µM 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 MicroBioSpin 6 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 μm 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, miR-196a, miR-196b 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% ethanal, 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 keratinocytes.

To identify miRNAs differentially expressed in various oral cancer cells and normal keratinocytes, we used a customized miRNA microarray (Agilent Technology, USA) which contained 470 human miRNA from the Registry. The raw miRNA signal histogram referenced by K6 sample was shown in Figure 1. In the Figure, the red bars indicate higher intensity and green bars indicate the lower intensity. The specific miRNA with same coloration may distribute differently in variant samples. The distinct pattern indicates that miRNA differently expressed in individual samples.

Total of 23 miRNAs most found significant differentially expressed between oral cancer cell lines and normal keratinocytes, and qPCR validation of miR-10b

After normalization and filtering to exclude the weak signal, 192 miRNAs were selected for clustering analysis as shown in the Figure 2. 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 HNC cells migration/invasion without affecting cell proliferation and have minima effect on HNC 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 4A, 4B). 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 5A). 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 5B, 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 HNC 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 6A, 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 6B). It was suggested that miR-10b had no significant function on radio-sensitivity of head-neck cancer cells.
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 regulates HNC 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].
References


Figure 1. Distinct miRNA expression signals between 10 samples.
Figure 2. (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 HNC 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 4. (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.

(A)

(B)
**Figure 5.** (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 μl 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 6. (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.