Profiling of MicroRNA Expression in Head and Neck Cancer

Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play regulatory roles by repressing translation or cleaving RNA transcripts. Accumulating evidence suggests that miRNAs are aberrantly expressed in many human cancers and that they play significant roles in the initiation, development and metastasis of human cancers. Genome-wide miRNA expression profiling provides information on the aberrant expression of miRNAs in cancers rapidly and precisely. Many human cancers have been profiled but there are few published studies in head and neck cancer. In this study, the expression of miRNAs was determined in five patient-matched head and neck tissues using miRNA microarray. Expression of eight miRNAs including miR-1, miR-126-1, miR-126-3p, miR-139, miR-375, miR-181a, miR-181b, miR-21 and miR-7 were validated using real-time RT-PCR and/or Northern blot analysis. This study provides the largest genome-wide survey of mature miRNA transcripts in head and neck cancer. The implication of aberrantly expressed miRNAs in head and neck cancer suggested their involvement in the development and progression of these malignancies. Further experiments of these deregulated miRNAs will help elucidate their roles in tumorigenesis and furnish their potential clinical usage for early diagnosis, prognosis and therapy of head and neck cancer.

Keywords: miRNA; Head and neck cancer; Cancer genetics

Abbreviations: HN: Head and Neck; HPV: Human Papilloma Virus; SCC: Squamous Cell Carcinomas; LCM: Laser Capture Microdissection; PGR: Photo Generated Reagent; FAK: Focal Adhesion Kinase

Introduction

Head and neck (HN) cancer is the sixth most common cancer worldwide, with approximately 650,000 new cases reported annually [1-3]. In the United States, there were 49,000 new cases in 2010, with 11,000 deaths [3,4]. Aggravating risk factors include tobacco smoking, alcohol consumption, betel chewing, and human papilloma virus (HPV) infection [3,5]. Head and neck cancer usually originates in the squamous cells lining the moist, mucosal surfaces inside the head and neck, such as inside the mouth, the nose, and the throat. All these squamous cell cancers are referred to as squamous cell carcinomas (SCC) of the head and neck. In spite of the overall incidence decrease of HNSCC in the United States during the past thirty years, there is a dramatic incidence increase for squamous cell malignancies of the base of tongue, and tonsil, especially in the populations who are young-to-middle-age patients. This is most likely coming from the rising incidence of HPV-associated HNSCC [6]. Although treatments of head and neck cancer include surgery, radiation therapy, chemotherapy, targeted therapy, or a combination of treatments, the 5-year survival rate for locoregionally advanced head and neck cancer patients is still poor [3,7-9]. The genetic alteration of cells in wide preneoplastic fields results in locoregional recurrence and second primary cancer. Half of all individuals still die from their disease. The characterization of the mechanisms involved in tumorigenesis and the identification of potential markers allow the identification of patients with biologically aggressive tumors, which are of great interest for the effective management of HNSCC patients.

MicroRNAs (miRNAs) are short non-coding RNAs identifying in plants, animals, and some viruses working in RNA silencing and post-transcriptional regulation of gene expression. While approximately 20% of human miRNAs are transcribed by RNA polymerase III, most of the miRNAs are transcribed by the RNA polymerase II to produce a primary-microRNA (pri-miRNA), which is then processed as ~60-nt long pre-miRNA hairpin intermediates. The intermediates are further processed to ~22-nt miRNA duplexes to negatively regulate protein expression by miRNA degradation or protein translation repression through base-pairing to complementary sites on the targets [10]. Mounting evidences have indicated the pivotal role of miRNAs in many biological processes including cell motility, cell proliferation, apoptosis, cell metabolism, cell differentiation, morphogenesis, and developmental timing [11,12]. Numerous studies have revealed the importance of miRNAs in carcinogenesis [13,14] and...
defined that high-throughput miRNA profiling is an informative tool to determine the lineage of development and the status of differentiation in various human malignancies. However, very little attention has been paid to head and neck cancer in genome miRNA analysis. Urgent priorities are needed to identify novel gene(s) or pathways that may elucidate the mechanisms of this disease. In this study, miRNA microarray was utilized to measure the relative expression of miRNAs in head and neck cancer. In addition, we attempted to seek associations in the literature between these miRNAs and their potential targets for a role in carcinogenesis of head and neck.

Materials and Methods

Patients and samples

The tissue samples (Table 1) were obtained from patients with head and neck cancer diagnosed by the pathologists in the First Affiliated Hospital of Anhui Medical University with informed consent and agreement. All specimens were snap frozen in liquid nitrogen immediately after surgical resection, and stored in -80°C until used. The study was approved by the medical ethics committee of Tsinghua University.

To obtain pure cancer and/or normal cells, laser capture microdissection (LCM) was carried out by the qualified pathologist. Total RNAs were extracted by using the miRvana™ miRNA Isolation Kit (Ambion, Austin, Texas) according to the instructions of the manufacturer. The RNA quality was evaluated by 15% denaturing polyacrylamide gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer, Eppendorf, Hamburg, Germany).

µParaflo™ MicroRNA microarray Assay

High-throughput miRNA gene expression analysis for five snap-frozen patient-matched head and neck samples (HN00587T/C, HN00647T/C, HN00653T/C, HN00719T/C and HN00728T/C) was executed by LC Sciences (http://www.lcsiences.com/; Houston, TX). The assay started from 2 to 5 µg total RNA sample, which was size-fractionated using a YM-100 Microcon centrifugal filter, and the small RNAs (< 300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a microcirculation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA from miRBase (http://microrna.sanger.ac.uk/sequences/) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using PGR [photogenerated reagent] chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µl 6xSSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After hybridization detection used fluorescence labeling using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner GenePix 4000B (Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics). Numerical intensities were extracted for control, background, and miRNA probes and converted into Microsoft Excel spreadsheets.

Table 1: Summary of human head and neck cancer tissues used in this study.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN00051</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00143</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00165</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00174</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00197</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00212</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00214</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00489</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00542</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00554</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00555</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00556</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00571</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00581</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00587</td>
<td>miRNA microarray, Northern blot, LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00596</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00607</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00647</td>
<td>miRNA microarray, Northern blot, LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00653</td>
<td>miRNA microarray, Northern blot, LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00654</td>
<td>LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00719</td>
<td>miRNA microarray, Northern blot, LCM, qRT-PCR</td>
</tr>
<tr>
<td>HN00728</td>
<td>miRNA microarray, Northern blot, LCM, qRT-PCR</td>
</tr>
</tbody>
</table>

Data Analysis

Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p-values of the t-test were calculated; differentially detected signals were those with less than 0.01 p-values. The TIGR MeV (Multiple Experimental Viewer) (the Institute for Genomic Research) microarray program was used for clustering analysis.

Real-time RT-PCR

Total RNAs were isolated using mirVana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, Texas) according to the instructions of the manufacturer. Twenty ng of total RNAs were used for reverse transcription of cDNA. The PCR reaction was performed in iCycler (Bio-Rad, Hercules, CA) consisting of appropriate number of cycles (95 °C for 15 s, 60 °C for 30 s) after an initial denaturation step (95 °C for 3 min) using mirVana™ qRT-PCR Primer Sets (Ambion, Austin, Texas). In addition, the PCR products were separated on 3.5% agarose gel and visualized with ethidium bromide on the ChemiImager Imaging System 5500 (Alpha Innotech, San Leandro, CA). The 5 S RNA was used as an internal control.

Northern blotting

Northern blotting was performed as previously described [15]. Briefly, 10 µg of total RNAs from each sample was fractionated on 15% denatured polyacrylamide gel containing 8M urea, then transferred onto Bright Star® charged nylon membrane (Ambion, Austin, Texas) using semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Blots were pre-hybridized at 65°C for 1 hour in pre-hybridization buffer (200 mM NaHPO₄, pH7.0, 5% SDS) and once for 10 min at 42°C using high-stringency buffer (1% SDS, 1×SSC). The blots were exposed to X-ray film (PIERCE, Rockford, IL) in -80°C freezer. The oligonucleotides used as probes are the complementary sequences of the mature miRNA (miR Registry, http://www.sanger.ac.uk/Software/Rfam/miRNA/); miR-181a: 5'-adccgacagcatgtaagtt-3'; and miR-21: 5'-taacttcagctgttaagatc-3'. An oligonucleotide complementary to the U6 RNA (5'-gcagggccatgctaatcttctctgtatcg-3') was used to normalize expression levels.

Results

Altered expression of miRNAs in head and neck cancer

Total RNAs from 5 pairs of snap-frozen patient-matched head and neck samples were sent to LC Sciences (Houston, TX) for miRNA microarray assay. The miRNA expression profiles were defined between tumor (designated as "T") and adjacent normal tissues (designated as "C") after performing the correlation analysis. By executing the correlation analysis from 2 chips, the system related biases coming from labeling, handling can be eliminated and therefore calls can be narrowed down to the true biological differences. Differentially expressed miRNAs (at P < 0.01) between tumor and adjacent normal tissues were identified. Table 2 showed the eight miRNA candidates chosen for further validation. Table 2 showed the unsupervised hierarchical clustering of miRNA expression.

The 5 paired samples are in columns and the miRNAs are in rows. T, tumor tissue; C, adjacent normal tissue.

Table 2: Eight miRNAs of interest chosen from the differentially expressed transcripts for validation.

<table>
<thead>
<tr>
<th>miRNA candidates</th>
<th>signal intensity</th>
<th>HNSFT00587 Tumor</th>
<th>HNSFT00587 Normal</th>
<th>HNSFT00647 Tumor</th>
<th>HNSFT00647 Normal</th>
<th>HNSFT00653 Tumor</th>
<th>HNSFT00653 Normal</th>
<th>HNSFT00719 Tumor</th>
<th>HNSFT00719 Normal</th>
<th>HNSFT00728 Tumor</th>
<th>HNSFT00728 Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>70553.63</td>
<td>19652.55</td>
<td>40717.68</td>
<td>25689.0</td>
<td>67534.99</td>
<td>21435.48</td>
<td>73021.53</td>
<td>12934.91</td>
<td>8230.68</td>
<td>8099.10</td>
<td></td>
</tr>
<tr>
<td>miR-7</td>
<td>735.92</td>
<td>222.63</td>
<td>_</td>
<td>_</td>
<td>1691.31</td>
<td>136.20</td>
<td>541.58</td>
<td>12.60</td>
<td>109.68</td>
<td>5.24</td>
<td></td>
</tr>
<tr>
<td>miR-181a</td>
<td>4170.48</td>
<td>1736.07</td>
<td>_</td>
<td>_</td>
<td>4057.30</td>
<td>1883.81</td>
<td>2119.26</td>
<td>763.45</td>
<td>2961.16</td>
<td>1319.05</td>
<td></td>
</tr>
<tr>
<td>miR-181b</td>
<td>3200.38</td>
<td>1088.88</td>
<td>1356.68</td>
<td>355.22</td>
<td>3030.89</td>
<td>1293.69</td>
<td>1603.81</td>
<td>584.52</td>
<td>3012.02</td>
<td>1166.77</td>
<td></td>
</tr>
<tr>
<td>miR-1</td>
<td>30.8</td>
<td>3458.69</td>
<td>488</td>
<td>577.67</td>
<td>58.15</td>
<td>1299.80</td>
<td>258.39</td>
<td>3137.54</td>
<td>2421.02</td>
<td>24670.59</td>
<td></td>
</tr>
<tr>
<td>miR-126*</td>
<td>92.78</td>
<td>3570.01</td>
<td>_</td>
<td>_</td>
<td>94.76</td>
<td>4008.85</td>
<td>475.66</td>
<td>3157.70</td>
<td>61.29</td>
<td>796.16</td>
<td></td>
</tr>
<tr>
<td>miR-139</td>
<td>7.38</td>
<td>270.99</td>
<td>_</td>
<td>_</td>
<td>18.64</td>
<td>425.76</td>
<td>28.35</td>
<td>171.23</td>
<td>11.22</td>
<td>313.75</td>
<td></td>
</tr>
<tr>
<td>miR-375</td>
<td>476.46</td>
<td>7529.95</td>
<td>91.4</td>
<td>356.57</td>
<td>286.86</td>
<td>5899.93</td>
<td>109.30</td>
<td>626.28</td>
<td>128.59</td>
<td>1620.45</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Unsupervised hierarchical clustering of miRNA profiles of 5 paired samples from head and neck cancer using Cluster 3.0.
Validation of the miRNA microarray

Eight differentially expressed miRNA candidates miR-1, -126* (-126-3p), -139, -375, -181a, -181b, -21 and -7 were chosen to confirm the miRNA microarray results by Northern blotting and/or real-time PCR. The results indicated that comparing their adjacent normal counterparts, miR-181a, -181b, -21, and -7 were upregulated in cancer, while miR-1, -126* (-126-3p), -139, and -375 were down-regulated in cancer (Figure 2). These results were consistent with the results from miRNA microarray assay.

Expression of miRNA candidates in laser capture microdissected (LCM) samples

Laser capture microdissection was performed for twenty-one patient-matched head and neck samples, and the total RNAs were extracted for real-time PCR. The results showed that, in comparison with their adjacent normal counterparts, miR-181a, -181b, -21, and -7 were increased in cancer cells while miR-1, -126* (-126-3p), -139, and -375 were decreased in cancer cells (Figure 3).

Real-time RT-PCR of miR-1, -126* (-126-3p), -139, -375, -181a, -181b, -21 and -7 in patient-matched laser capture microdissected head and neck samples. The 5S rRNA was used as an internal control. T, tumor tissue; C, adjacent normal tissue.

Discussion

Carcinogenesis is a complicated and multi-step process in which many genes are implicated [16]. MicroRNAs constitute a novel class of gene regulators that are ~22 nt small non-coding, single-stranded RNAs [17]. It has been found that they are abnormally expressed in diverse cancers [18-20]. And studies have shown that some miRNA signatures could be correlated well for the classifications with different clinicopathologic parameters [21,22]. To develop rational approaches for the diagnosis and treatment of cancer depends on identifying new biomarkers and understanding the molecular mechanisms that underlie tumor formation and progression. In this aspect, studies that seek to identify dysregulated miRNAs in neoplasm are critical. Thus, research efforts aimed at systematically identifying the miRNA expression profiles between normal and tumor cells are critically needed. In this study, we have defined the miRNA expression profiles in 5 patient-matched human head and neck samples using miRNA microarray. After data analysis, we obtained the differentially expressed miRNAs (P<0.01) between normal and cancer samples. The profiles of miRNAs from samples HN00587,
Recent advances in the understanding of the oncogenesis have revealed multiple deregulated miRNAs [23,24]. Most of the differentially expressed miRNAs in head and neck cancer we found showed are consistent with previous studies [25]. For example, miR-1, a highly conserved miRNA in the musculatures of flies, mice and humans and a well characterized as muscle-specific miRNA, which was downregulated in head and neck cancer from this study, was consistently down-regulated in human prostate tumors [26-28]. In colorectal cancer, miR-1 directly decreased MET oncogene both at RNA and protein level, and its recovery resulted in reduction of cell proliferation and motility driven by MET, identifying that miR-1 served as a tumor suppressor [29]. In gastric cancer, miR-1 inhibited cell proliferation and migration by targeting MET [30,31]. MiR-126* (miR-126-3p), miR-139, and miR-375. To confirm the results of the microarray analysis we performed northern blot and/or real-time PCR on a limited number of samples. Northern blotand/or qRT-PCR confirmed the results obtained by microarray analysis.

Genetic changes are the key elements to the development of cancer. And these genetic changes endow the cancer cells with many of the hallmarks of cancer, such as self-sufficient growth and resistance to anti-growth and pro-death signals [3]. Although mounting evidences have shown that genetic changes occur within cancer cells themselves, such as activated oncogenes or malfunctioned tumor suppressors, are responsible for many aspects of cancer development, it is likely that tumor progression is through multiple mechanisms that are not yet fully understood. One possible mechanism is that tumor promotion and progression are dependent on physiologic responses provided by supportive tissues of the tumor environment but that are not necessarily cancerous themselves. To verify whether the dysregulation of miRNAs was effectively restricted to the tumor cells in the samples, laser capture microdissection was performed to obtain pure cancer cells and normal cells for real-time PCR. As shown in Figure 3, real-time PCR results also confirmed the dysregulation of miRNAs cancer samples compared to normal samples. So far this is one of the first studies of this nature in head and neck cancer tissues.

Conclusion

In summary, the goal of this study was to profile the relative expression of miRNAs in head and neck cancer samples. How the miRNAs of interest play in cancer remains mostly unknown, and their clinical utility needs to be determined. However, these findings provide a base foundation for further characterization of miRNAs functions in head and neck cancer.

References


