MicroRNA expression profiles in supraglottic carcinoma

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Abstract. MicroRNAs (miRNAs) are single-stranded RNA molecules which regulate gene expression at the post-transcriptional level and several miRNAs have been found to be associated with some types of cancer. We sought to identify the expression and involvement of miRNAs in supraglottic carcinoma tissues compared with normal tissues and to determine whether miRNA expression is predictive of disease. Unsupervised clustering shows that miRNA profiles can distinguish tumor from normal tissues. Analysis of miRNA contents in supraglottic carcinoma highlighted nineteen differentially expressed miRNAs, three upregulated miRNAs (miR-21, miR-19a, miR-33a) and two downregulated miRNAs (miR-206, miR-375). The microarray results of supraglottic carcinoma and related computer analysis may be beneficial for further analysis of cancer diagnosis and therapy.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a very common malignant neoplasm of the head and neck. One of the key challenges in the treatment of LSCC is the management of metastasis to locoregional lymph nodes. Lymph node metastasis is common in supraglottic carcinoma and is the main cause of mortality for these patients. It has been reported that the incidence of lymph node metastasis may be as high as 25-50% in supraglottic carcinoma (1). Current treatments, including surgical intervention, radiation therapy and chemotherapy, have a moderate effect on early-stage cases, but are less effective in more advanced cases. Five-year overall survival for supraglottic cancer remains poor (2,3). Therefore, understanding the molecular pathways of carcinogenesis or progression is key to improving diagnosis, therapy and prevention of supraglottic carcinoma.

microRNAs (miRNAs) are a class of small, non-coding RNAs that are endogenously expressed in animal and plant cells. They regulate the expression of protein-coding genes at the translational level. One strand of the mature double-stranded miRNA is incorporated into the RNA-induced silencing complex, which downregulates target mRNAs either by degradation or by translational inhibition (4). miRNAs play important roles in normal regulation of gene expression for developmental timing, cell proliferation and apoptosis. Moreover, altered miRNA expression is implicated in cancer. Recently, miRNA genes were implicated in several types of cancer (5-7). The expression of miRNAs varies between cancer and normal cells and it also varies among different types of cancer. It has been shown that some miRNAs are aberrantly expressed in several different types of cancer (8-10), suggesting that they may play a role as a novel class of oncogenes or tumor-suppressor genes.

Several target genes have been experimentally identified for some miRNAs in various LSCCs; however, the global pattern of cellular functions and pathways affected by miRNAs in supraglottic cancer remains elusive. In the present study, expression profiling of miRNAs in clinical supraglottic carcinoma tissue samples was carried out, revealing the relationship between miRNA expression and supraglottic carcinoma. We also studied the candidate expression of genes that regulate supraglottic carcinoma miRNA processing and identified the possible gene ontology, pathway, evolution relationship of these candidate target genes. This information may be the basis of identifying a clinically applicable diagnostic tool in the future.

Materials and methods

Laryngeal carcinoma specimens and RNA isolation. Ten supraglottic carcinoma specimens and ten adjacent normal tissues were collected by surgical resection from the First Affiliated Hospital of Harbin Medical University between January and December 2008. These specimens were from patients between 42 to 78 years old, including 6 males and

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4 females. Lymph metastasis was found in three cases. Prior to surgery, no patients were treated by radiotherapy or chemotherapy and they were in good condition without cancer transmission found from other parts of their bodies.

The samples were snap-frozen in liquid nitrogen and stored at -80˚C. Total RNA was isolated from adjacent normal tissue and tumor tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was quantified using the BioPhotometer (Eppendorf), aliquoted, and stored at -80˚C briefly until needed.

Microarray profiling. miRNA was synthesized, amplified and purified using the Illumina TotalPrep RNA Amplification kit (Ambion Inc.), following the manufacturer's recommendations. Total RNA (500 ng) was sent for miRNA profiling studies using the Human miRNA BeadChip (V.12.0) in Illumina BeadStation 500GX (Illumina, Inc.), which is single-channel format according to the standard operating procedures of the company. RNA was reverse transcribed. After second strand synthesis, the cDNA was transcribed *in vitro* and ncRNA labelled with biotin-16-UTP. Labelled probe hybridization to BeadChip was carried out. Briefly, poly-A tail was added, cDNA synthesis (biotinylated) followed, combined with miRNA specific oligo, then extension and ligation. PCR (Mastercycler 5333), lable, hybridization (45˚C, 14-20 h), wash and imaging were carried our. Human miRNA BeadChip (V.12.0) contains 1,145 probes specific to human miRNA assessed at ~30 different beads on average (~850 probes from Sanger miRBase V.12.0 and 296 from literature or novel content identified with Illumina Solexa sequencing). The Cy3 fluorescence on the arrays was scanned at an excitation wavelength of 532 nm using a BeadArray Reader GX scanner (Illumina). Illumina BeadStudio software (version 1.5.0.34) was used for preliminary data analysis. Several quality control procedures were implemented to assess the quality of the whole experiment. Total RNA control samples were analyzed in the process. The Illumina BeadStudio software was used to view control summary reports and scatter plots. The scatter plots indicated a reduction in assay performance and highlighted samples that were of lower quality. The control summary report is generated by the BeadStudio software, which evaluates the performance of the built-in controls of the BeadChips in the process. This allows the user to look for variations in signal intensity, hybridization signal, background signal and the background to noise ratio for all samples analyzed in that run. Data are expressed as log2 ratios of fluorescence intensities of the experimental and the common reference sample. The Illumina data were then normalized using the 'normalize quantiles' function in the BeadStudio software.

miRNA expression analysis. Cluster analysis was performed using the metric Euclidean distance to compute the distance matrix for supraglottic carcinoma patients based on the expression of 1,145 differentially expressed miRNAs. To agglomerate the patients in the hierarchical cluster, we used the Ward method. Function heatmap.2 from R package gplots was used for the graphical display of the dendrogram (11).

Predicted miRNA target analysis. Potential miRNA targets were predicted and analyzed using DIANA TarBase (12) (http://diana.pcbi.upenn.edu/). Predicted target lists were analyzed using DIANA mirPath for association with molecular pathways potentially altered by the expression of single or multiple miRNAs (13). Predicted target lists were also analyzed for association with Gene Ontology (GO) (14) terms using L2L microarray analysis tools (15).

Table I. Differentially expressed miRNAs in supraglottic carcinoma tissues compared with normal tissues.

miRNA	Chromosome	Expression fold (cancer/normal tissue)	P-value
miR-34b	11	0.061481	7.36E-38
miR-885-5p	3	0.107502	7.36E-38
miR-218	4,5	0.133269	7.36E-38
miR-34c-3p	11	0.133316	7.36E-38
miR-299-5p	14	0.134148	7.36E-38
miR-375	2	0.139267	7.36E-38
miR-30a	6	0.140864	3.62E-33
miR-206	6	0.148176	2.83E-10
miR-410	14	0.16099	2.15E-27
miR-1299	9	0.16614	6.60E-25
miR-128a:9.1	-	0.168493	7.36E-38
miR-432	14	0.170929	6.93E-37
miR-136	14	0.173921	6.49E-13
miR-486-3p	8	0.182158	1.16E-36
miR-337-3p	14	0.186334	7.86E-35
miR-125b-2	21	0.187213	3.62E-20
miR-21	17	8.453834	0.008087
miR-19a	13	13.15016	0.0054576
miR-33a	22	13.33146	0.0057371

The miRNA microarray analysis was performed to detect the differentially expressed miRNAs in supraglottic carcinoma and normal tissues. P-values were generated in BeasStudio Software. miRNA, microRNA.

Evolution analysis. Potential miRNA target sequences were obtained from the National Center for Biotechnology Information website (NCBI, USA). The sequences were aligned by the ClustalW program (http://www.ebi.ac.uk/clustalw/) (16). The evolutionary tree of protein sequences was constructed by the neighbor-joining (NJ) method with protein p-distances by MEGA4 (Molecular Evolutionary Genetics Analysis) software (http://www.megasoftware.net/index.html) (17) and the reliability of the tree topology was assessed by 1,000 bootstrap replications.

Results

miRNA expression profile in supraglottic carcinoma and normal tissues. Using the miRNA microarray containing 1,145 human miRNA probe sets, we first assessed the miRNA profiles in three paired supraglottic carcinoma and normal tissues.

Unsupervised hierarchical clustering based on all the miRNAs spotted on the chip revealed a marked, distinct

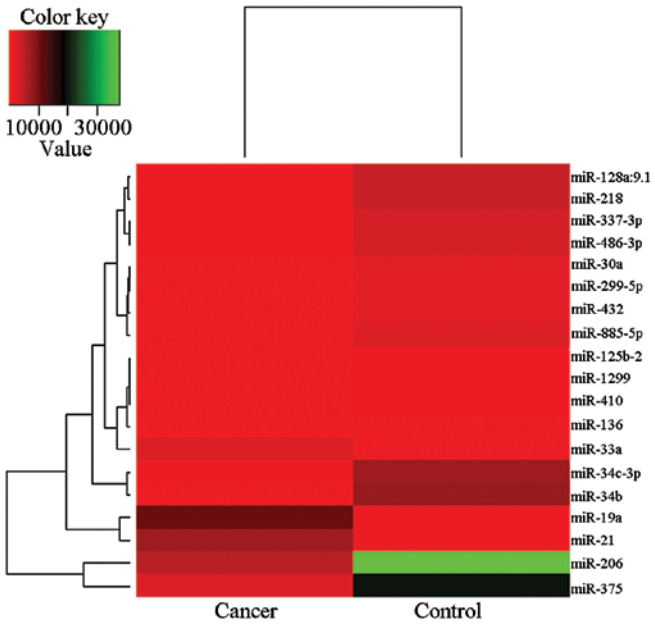

Figure 1. Differentially expressed miRNAs between supraglottic carcinoma and normal tissues. Results of the unsupervised hierarchical clustering analysis between groups of cancer tissues and normal tissues based on differential expression of 19 significantly altered miRNAs. The heat map shows relative levels of miRNA expression in a red (low relative expression) to green (high relative expression) scale. miRNA, microRNA.

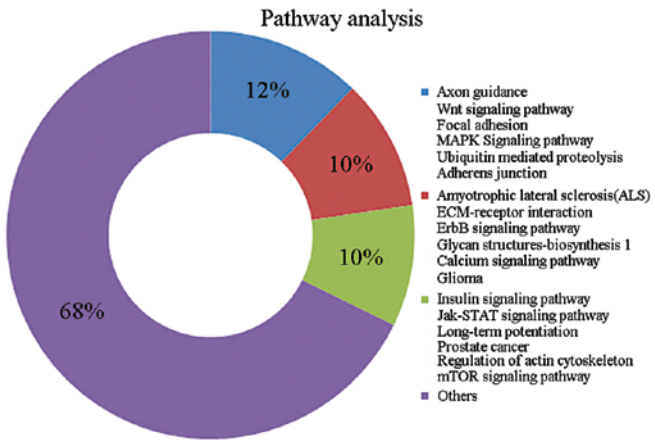

Figure 2. Potential miRNA target gene pathway analysis in supraglottic carcinoma. miRNA, microRNA.

separation of the supraglottic carcinoma miRNA profiles compared with those of normal tissues. Significance analysis of microarray identified 85 significantly differentially expressed miRNAs (P<0.01) between supraglottic carcinoma and normal tissues. These human miRNAs further identified by significance analysis of microarray method showed >5-fold difference between supraglottic carcinoma and normal tissues. This method identified 19 miRNAs to be significantly altered in their expression between supraglottic carcinoma and non-diseased tissues, with 3 being upregulated (miR-21, miR-19a, miR-33a) and 16 downregulated in supraglottic carcinoma (Table I). Unsupervised clustering analysis using these 19 miRNAs discriminated cancer from non-cancer tissues (Fig. 1), thus indicating that these miRNAs may be a

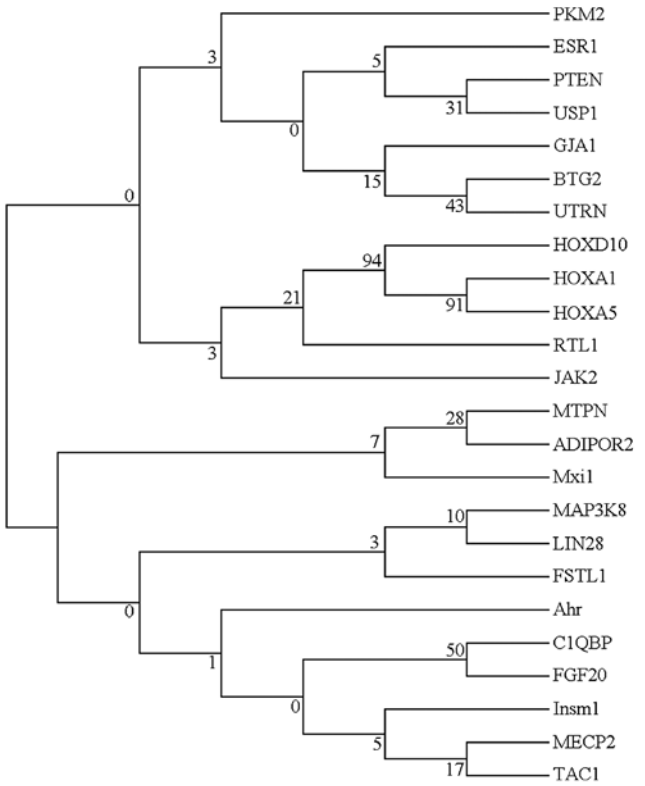

Figure 3. Evolutionary tree and sequence divergences of potential miRNA target genes. The values on each node are percentage of bootstrap values. miRNA, microRNA.

valid diagnostic for supraglottic carcinoma, although due to the small number of samples analyzed, this requires further analysis.

Bioinformatics analysis of supraglottic carcinoma miRNA target genes. To investigate the involvement of miRNAs in supraglottic carcinoma, we performed systemic bioinformatics analysis to identify potential gene targets, including pathway analysis (Fig. 2). Several pathways were enriched in the target genes. Notably, multiple genes were related to signaling pathways. The list of regulated gene targets was also used to perform a GO (14) analysis to evaluate the specific functional categories of genes from broad GO categories (Table II). The results indicated that seven biological processes, six molecular functions and two cellular components were statistically enriched with these miRNA targets. Furthermore, we found that 25 potential target genes related to 12 miRNAs which were strongly supported by references to avoid the false negative predictions (Table III). As a result, 3 target genes were involved in mRNA cleavage and 13 target genes were involved in mRNA repression. The evolutionary tree of miRNA target genes was further analyzed to illustrate the function of these genes (Fig. 3). These genes showed a high level of sequence divergence. It is reasonable to infer that these genes are members of a multigene family, since they shared low sequence similarity. Generally, a node with >70% bootstrap supporting value is a stable node. The NJ-tree of miRNA target genes exhibited low bootstrap supporting values on each deep branch node and, therefore, few strong conclusions may be drawn from the evolutionary tree.

Table II. Gene Ontology analysis in supraglottic carcinoma.

GO type	GO term	Description	P-value
Process	GO: 0006725	Cellular aromatic compound metabolic process	2.79E-4
Process	GO: 0050776	Regulation of immune response	4.64E-4
Process	GO: 0001919	Regulation of receptor recycling	4.94E-4
Process	GO: 0003015	Heart process	5E-4
Process	GO: 0060047	Heart contraction	5E-4
Process	GO: 0019882	Antigen processing and presentation	5.76E-4
Process	GO: 0006066	Alcohol metabolic process	8.35E-4
Process	GO: 0008152	Metabolic process	9.43E-4
Process	GO: 0031943	Regulation of glucocorticoid metabolic process	9.83E-4
Function	GO: 0005488	Binding	5.05E-7
Function	GO: 0003824	Catalytic activity	1.08E-5
Function	GO: 0004423	Iduronate-2-sulfatase activity	5.37E-4
Function	GO: 0046872	Metal ion binding	5.9E-4
Function	GO: 0000062	Acyl-CoA binding	9.21E-4
Function	GO: 0043169	Cation binding	9.35E-4
Component	GO: 0044424	Intracellular part	3.63E-6
Component	GO: 0044444	Cytoplasmic part	8.87E-5

Gene Ontology (GO) analysis was performed to detect the differentially expressed miRNAs in supraglottic carcinoma and normal tissues. P-values were calculated from binomial distribution. miRNAs, microRNAs.

Table III. Comparison of pathways associated with miRNA and mRNA signatures.

miRNA	Gene	Type	Author/(Ref.)
miR-10a	HOXA1	mRNA repression	Garzon *et al* (29)
miR-10b	HOXD10	mRNA repression	Ma *et al* (33)
miR-125b	LIN28	mRNA cleavage	Wu and Belasco (39)
miR-133a	PKM2	Unknown	Wong *et al* (38)
miR-133b	PKM2	Unknown	Wong *et al* (38)
miR-136	RTL1	mRNA cleavage	Davis *et al* (28)
miR-19a	MECP2	Unknown	Lewis *et al* (32)
miR-19a	PTEN	mRNA repression	Lewis *et al* (32)
miR-19a	HOXA5	Unknown	Lewis *et al* (32)
miR-206	FSTL1	mRNA repression	Rosenberg *et al* (36)
miR-206	UTRN	mRNA repression	Rosenberg *et al* (36)
miR-206	GJA1	mRNA repression	Anderson *et al* (27)
miR-206	ESR1	mRNA cleavage	Adams *et al* (26)
miR-206	TAC1	mRNA repression	Greco and Rameshwar (30)
miR-370	MAP3K8	Unknown	Meng *et al* (34)
miR-375	MTPN	mRNA repression	Poy *et al* (35)
miR-375	Ahr	Unknown	Krek *et al* (31)
miR-375	C1QBP	mRNA repression	Krek *et al* (31)
miR-375	Insm1	Unknown	Krek *et al* (31)
miR-375	ADIPOR2	mRNA repression	Krek *et al* (31)
miR-375	JAK2	mRNA repression	Krek *et al* (31)
miR-375	Mxi1	Unknown	Krek *et al* (31)
miR-375	USP1	mRNA repression	Krek *et al* (31)
miR-433	FGF20	Unknown	Wang *et al* (37)
miR-21	BTG2	mRNA repression	Liu *et al* (22)

Pathway analysis was performed to detect the differentially expressed miRNAs in supraglottic carcinoma and normal tissues. miRNAs, microRNAs.

Discussion

The present study demonstrated that multiple microRNAs (miRNAs) are upregulated or downregulated in supraglottic carcinoma, including an extensively validated subset that may be potential clinical biomarkers of disease. Microarray profiling of more than 1,000 miRNAs identified 85 miRNAs that were significantly differentially expressed in tumor tissues compared with normal tissues. Of the 85 human miRNAs, three upregulated miRNAs (miR-21, miR-19a, miR-33a) and two downregulated miRNAs (miR-206, miR-375) were highlighted. Twenty-five target genes were identified to help characterize the diverse functions of miRNAs. Furthermore, biology process and evolution were explored to find the function of miRNAs.

miRNA expression profiles can be used to classify human cancer (14,18). Distinct signatures for several epithelial cancers have been reported, such as breast, lung, pancreatic and gastric cancer (9,19-21). Our study explored miRNA expression in supraglottic carcinoma. Consistent with previous reports of miRNA expression in head and neck cancer (22-25), some miRNAs were significantly deregulated, although changes in individual miRNAs did not match completely. For example, miR-21 was significantly upregulated in all head and neck cancer analysis (22-25). Tran *et al* found miR-21 and miR-205 were highly expressed in head and neck cancer cell lines (25). Chang *et al* found miR-21, let-7, 18, 29c, 142-3p, 155 and 16b were significantly overexpressed in primary head and neck squamous cell carcinoma (24). Avissar *et al* found miR-221 and miR-375 were significantly altered and upregulated in head and neck squamous cell carcinoma (23). Liu *et al* identified 13 miRNAs that were differentially expressed, 7 miRNAs were downregulated and 6 miRNAs (let-7a-1, miR-203, miR-205, miR-21, miR-98 and miR-16-1) were upregulated in laryngeal carcinoma tissues based on 210 human miRNA probe sets (22). This indicated that the molecular biology of head and neck squamous cell carcinoma is complex. It is important to analyze the miRNA mechanism on a larger scale.

miRNAs modulate gene expression by targeting mRNAs for translational suppression or mRNA cleavage and it is well known that miRNAs regulate a variety of cellular activities through their effect on the expression of multiple target genes (4). The identification of target genes regulated by a specific miRNA has been proved difficult despite the development of computational approaches to predict miRNA targets. The ability to find target genes is further complicated by the fact that target selectivity of miRNAs may depend on the cellular microenvironment. Our studies identified 25 reliable target genes, supported by previous studies (22,26-39). The evolutionary tree of these target genes showed a low sequence similarity and it indicated that miRNAs may play different roles in supraglottic carcinoma. Also, these results provided a possible way to address biological meaning from the global pattern of cellular functions and pathways that are affected by miRNAs in supraglottic carcinoma. Gene Ontology (GO) (14) was developed into three structured controlled vocabularies (ontologies) that describe gene products in components and molecular functions in a species-independent manner (40). GO enrichment analysis was used to reduce the number of targets of a large group of co-expressed miRNAs and to find biological functions potentially affected by multiple miRNAs in our research. We performed a statistical enrichment analysis of GO categories to find categories that are enriched with targets of co-expressed miRNAs. It allowed us to reduce a very large raw list of predicted target genes to a smaller set of target genes from significantly enriched GO categories. We assumed that filtering GO categories on the total number of hits by miRNAs targeting the same category would reduce the number of false positive target predictions and at the same time would allow narrowing down of the large target lists and determining those biological functions.

In conclusion, our global analysis of miRNA array in human supraglottic carcinoma tissues demonstrated that co-expressed miRNAs may collectively provide systemic compensatory response to the abnormal functional and phenotypic changes in supraglottic carcinoma by targeting a broad range of functional categories and abnormally activated pathways known to be affected in supraglottic carcinoma. Such system biology based approach may provide new avenues for biological interpretation of miRNA profiling data as well as generation of experimentally testable hypotheses regarding collective regulatory functions of miRNAs in supraglottic carcinoma biology.

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