Integrated analysis of deregulation microRNA expression in head and neck squamous cell carcinoma

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Abstract

MicroRNAs (miRNAs) play critical roles in carcinogenesis and development of cancers. In this study, we analyzed the eccentrically expressed miRNAs in head and neck squamous cell carcinoma (HNSCC) tissues based on the miRNA-Seq data of HNSCC patients available in the Cancer Genome Atlas database. Aberrant expression of 2589 miRNAs was detected in HNSCC tissues (1128 downregulated and 1461 upregulated). The differential expression levels of the miRNAs were further validated by analysis of 25 HNSCC samples and paired control tissues and compared with the Gene Expression Omnibus database to determine the candidate miRNAs. Quantitative reverse transcription polymerase chain reaction was used to compare the expression of these candidate miRNAs between 22 fresh HNSCC tissue samples and 11 control samples. In addition, the relationship between the expression of these candidate miRNAs and Tumor, Node, Metastases staging of HNSCC was analyzed. Compared with the expression in control tissues, the levels of hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p were significantly lower in HNSCC. According to the Cancer Genome Atlas dataset analyzed, all 4 miRNAs were shown to inhibit tumor progression (T stage), positive lymph node metastasis (N stage), and distant metastasis (M stage) in HNSCC. Kyoto Encyclopedia of Genes and Genomes analysis showed that genes regulated by these 4 miRNAs were enriched in certain pathways, including the transforming growth factor-\(\beta\) signaling pathway and the Hippo pathway. Enriched gene ontology terms mainly included regulation of transcription, cell proliferation, and apoptosis, which are well-characterized functions of miRNAs. Moreover, all 4 miRNAs inhibited the progression of primary tumors (T stage) and metastasis of regional lymph nodes (N stage). The top 4 aberrantly expressed miRNAs identified in this study have great clinical value in developing strategies for early diagnosis and treatment of HNSCC. More intensive studies are required to elucidate the mechanism underlying the roles of these miRNAs in HNSCC.

Abbreviations: GEO = Gene Expression Omnibus, GO = Gene Ontology, HNSC = head and neck squamous cell carcinoma, KEGG = Kyoto Encyclopedia of Genes and Genomes, PPI = protein-protein interaction analysis, ROC = receiver-operating characteristic, TGCA = the Cancer Genome Atlas.

Keywords: Gene Expression Omnibus, head and neck squamous cell carcinoma, Kyoto Encyclopedia of Genes and Genomes, microRNA, the Cancer Genome Atlas, Tumor, Node, Metastases staging

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor internationally, comprising tongue (TSCC), oral (OSCC), laryngeal (LSCC), and nasopharyngeal (NPC) forms of the disease.\cite{1} With the development of industrialization and increased air pollution, there are approximately 600,000 new cases worldwide each year. Furthermore, approximately 80% to 90% of patients with HNSCC are reported to have a long history of smoking and drinking and some have a history of papilloma virus infection.\cite{2-4} Standard treatment strategies for HNSCC include surgery, radiation, and chemotherapy.\cite{5} Clinical symptoms of HNSCC vary in the early stage of the tumor development due to the heterogeneity of the disease. Therefore, identification of biomarkers of HNSCC is of great significance for improving the early diagnosis and treatment of patients.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs (approximately 22 nucleotides). Like long non-coding RNAs and protein-coding RNAs, miRNAs control the translation of target genes by direct binding to the 3'- or 5'-untranslated region of the transcribed mRNAs\cite{6} and play important roles in various biological processes, such as cellular development, metabolism, and proliferation.\cite{7} In particular, miRNAs are implicated in the pathogenesis and progression of cancer\cite{8}; thus,
the value of miRNAs as potential cancer biomarkers has become a focus of research. Many types of RNA (coding and noncoding) are aberrantly expressed in HNSCC patients. For instance, miR-26, miR-107, miR-125b, and miR-203 have been reported to be prognostic biomarkers of overall survival of HNSCC patients. However, the role of miRNAs in this type of cancer remain to be fully elucidated.

In recent decades, systematic analysis of the genome, transcriptome, and proteome datasets has become powerful tools for the discovery and validation of tumor markers. In this study, we investigated the molecular pathogenesis of HNSCC based on the miRNA-sequencing data of HNSCC patients available in the Cancer Genome Atlas (TCGA). We identified 4 miRNAs as a novel signature of the occurrence and progression of HNSCC, which offers the potential for the development of effective and individualized treatments for HNSCC patients.

2. Materials and methods

2.1. Identification of HNSCC TCGA dataset

TCGA database of HNSCC was searched following the publication guidelines as described previously. High throughput RNA sequencing data for HNSCC were downloaded from TCGA database. Generally, 528 HNSCC samples and 44 adjacent normal tissues with miRNA-Seq data from the Illumina HiSeq platform were included in current study. Approval by a local ethics committee was not required since TCGA is a community resource project and all data can be used for publication without restrictions or limitations.

2.2. Analysis of the aberrantly expressed miRNAs in HNSCC

The HNSCC miRNA-Seq dataset consists of 10,034 miRNAs defined by miRbase (http://www.mirbase.org/). GDCRNA Tools were used to detect differentially expressed genes between HNSCC and normal samples, and the adjusted P-value and log2FC were calculated. Genes that met the cutoff criteria of adjusted \( P < 0.05 \) and log2FC > 1.0 were considered to be differentially expressed genes. Then, the 21 most differentially expressed miRNAs were picked out when we set adjusted \( P < 0.01 \) and \( \text{log}_2 \text{FC} > 1.5 \). The area under the receiver-operating characteristic curve was used to assess the diagnostic accuracy of all the aberrantly expressed miRNAs, with 1 indicating a perfect discriminatory value and 0.5 or less indicating no discriminatory value. The 4 most differentially expressed miRNAs (hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p) with AUC > 0.80 were selected for further analysis of their potential roles and diagnostic value in carcinogenesis. Then, Tumor, Node, Metastases (TNM) stage of HNSCC patients was analyzed in relation to the expression of these 4 miRNAs from TCGA clinical data (Table 1).

2.3. Identification of the functions of aberrantly expressed miRNA in HNSCC

The co-expressed gene modules regulated by these 4 miRNAs were identified by protein-protein interaction analysis (PPI) using miRTarBase 7.0 and then visualized by Cytoscape 3.5.1. In addition, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the 4 miRNAs and their co-expressed genes were performed based on the Database for Annotation, Visualization, and Integrated Discovery (https://david.ncifcrf.gov/). The results with Benjamini–Hochberg adjusted \( P \)-values \(< 0.05 \) were considered statistically significant.

2.4. Validation of the aberrantly expressed miRNAs based on the Gene Expression Omnibus (GEO) database

The differential expression levels of the miRNAs were further validated by analysis of 25 HNSCC samples and paired control tissues using the GEO database (GSE34496).

2.5. Validation based on clinical HNSCC samples by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The data from TCGA and GEO databases were further validated by qRT-PCR analysis of hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p expression levels in 22 fresh HNSCC tissue samples (11 laryngocarcinoma, 4 paranasal sinus carcinoma, 3 nasopharyngeal carcinoma, 2 oropharyngeal carcinoma, and 2 hypopharyngeal carcinoma) and 11 nasopharyngeal epithelium control samples. All samples were reviewed by pathologists to confirm the diagnosis. The general clinical information for these patients is listed in Table 2. Patients who had received preoperative treatment (chemotherapy or radiotherapy) and those with local recurrences and second primary tumors were excluded from the study. Total RNA was

| Table 1: The clinical data of HNSCC patients from TCGA database. |
|-------------------|-------------------|-------------------|
| Characteristic    | Cases             | Percentage (%)    |
| Gender            |                   |                   |
| Male              | 386               | 73.1              |
| Female            | 142               | 26.9              |
| Age               |                   |                   |
| <50 yr            | 84                | 15.9              |
| 50–70 yr          | 336               | 63.6              |
| >70 yr            | 108               | 20.5              |
| Primary site      |                   |                   |
| Tongue            | 156               | 29.5              |
| Larynx            | 117               | 22.2              |
| Floor of mouth    | 56                | 10.6              |
| Tonsil            | 46                | 8.7               |
| Gum               | 11                | 2.1               |
| Other parts of mouth | 43        | 8.1               |
| Oropharynx        | 10                | 1.9               |
| Hypopharynx       | 9                 | 1.7               |
| Other sites       | 80                | 15.2              |
| T stage           |                   |                   |
| Tx                | 12                | 2.3               |
| T1-T2             | 191               | 36.2              |
| T3-T4             | 325               | 61.5              |
| N stage           |                   |                   |
| Nx                | 18                | 3.4               |
| M0                | 246               | 46.6              |
| N1-N3             | 264               | 50.0              |
| M stage           |                   |                   |
| Tx                | 21                | 4.0               |
| M0                | 496               | 93.9              |
| M1                | 11                | 2.1               |

HNSCC = head and neck squamous cell carcinoma, TCGA = the Cancer Genome Atlas.
extracted from clinical specimens using TRizol reagent (Thermo Fisher Scientific: Rockford, IL). The SYBR II Prime Script miRNA RT-PCR Kit (Takara, Mountain View, CA) was used for reverse transcription and real-time PCR following the manufacturer’s instructions. U6 was used as an internal control for miRNA detection. The relative expression levels of target genes were calculated using the \( \frac{2^{-\Delta \Delta CT}}{c} \) method. The qRT-PCR primers used are shown in Table 3. The relationship between the expression of these 4 miRNAs and TNM staging of HNSCC was analyzed.

The present study was approved by the ethics committee of Renmin Hospital of Wuhan University (WDRY2019-K058). All participants provided informed consent and agreed to the use of their clinical samples for research purposes.

### 2.6. Statistical analysis

Data were presented as the mean ± standard deviation and all statistical analyses were performed using SPSS version 22.0 (IBM).
Differential expression of miRNAs was analyzed by Student t test. P-values $<.05$ were considered to indicate statistical significance.

3. Results

3.1. Screening candidate miRNAs based on the TCGA-HNSCC dataset

Aberrantly expressed miRNAs in HNSCC were identified by systematic analysis of the miRNA expression levels in 523 HNSCC samples and 44 adjacent normal tissues obtained from the TCGA dataset using the R package DEseq. In total, we identified 2589 aberrantly expressed miRNAs (1128 downregulated and 1461 upregulated) in HNSCC tissues (Fig. 1). Four miRNAs with the greatest differential expression (hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p) were selected for further analysis of their potential roles in carcinogenesis and diagnostic value. These 4 miRNAs had an area under the receiver-operating characteristic curve (AUC) exceeding 0.80 (Fig. 2) and their characteristics are shown in Table 4.

3.2. Clinical value of the 4 aberrantly expressed miRNAs in patients with HNSCC

All 4 miRNAs were expressed at markedly lower levels in HNSCC compared with those in adjacent normal tissues (Fig. 3). Hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p inhibit tumor progression (T stage), positive lymph node metastasis (N stage), and distant metastasis (M stage) in HNSCC ($^P<.05$, $^{**}P<.01$, Fig. 4).

3.3. Validation of the 4 aberrantly expressed miRNAs using the GEO dataset

The top 4 aberrantly expressed miRNAs were validated by analysis of 25 HNSCC samples and paired control tissues using the GEO database (GSE34496). Compared with the paired adjacent control tissues, the levels of all 4 miRNAs were notably lower in the HNSCC tissues ($P<.05$) (Fig. 5A and B).

3.4. Potential mechanism by which the 4 aberrantly expressed miRNAs regulate the development and progression of HNSCC

To investigate the potential functions of the top 4 aberrantly expressed miRNAs and their value in predicting HNSCC occurrence, we first identified a total of 2063 genes targeted by these miRNAs. Functional enrichment analysis showed significant enrichment of these genes in 40 GO processes (adj. $P<.01$). The top 12 enriched GO terms are shown in Figure 6A. The most commonly enriched GO terms were regulation of transcription, cell proliferation, and apoptosis, which are known functions of miRNAs. Further analysis showed significant enrichment in 11 KEGG pathways (adj. $P<.01$), including several cancer-related pathways, such as the transforming growth factor-$\beta$ (TGF-$\beta$) signaling pathway and Hippo signaling pathway. The top 7 enriched KEGG pathways are shown in Figure 6B.

PPI of the top 4 aberrantly expressed miRNAs revealed 15 genes that were co-expressed with hsa-miR-410-3p, 16 genes that were co-expressed with hsa-miR-411-5p, 18 genes that were co-expressed with hsa-miR-125b-2-3p, and 8 genes that were co-expressed with hsa-miR-99a-3p (Fig. 7). These results indicated an association of the top 4 aberrantly expressed miRNAs with regulation of gene expression and critical cell functions.

3.5. Verification of the expression level of dysregulated genes in tumor tissues from patients with HNSCC

The RNA-Seq data were validated by qRT-PCR analysis of the expression levels of the 4 aberrantly expressed miRNAs in tumor tissues from patients with HNSCC ($n=22$) and control samples ($n=11$). Compared with the expression levels in the control tissues, hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p...
and hsa-miR-99a-3p were significantly downregulated in tumor tissues samples from patients with HNSCC (Fig. 8A). These 4 miRNAs inhibited tumor progression (T stage), positive lymph node metastasis (N stage) in patients with HNSCC (Fig. 8B, \*P < .05, \*\*P < .01). These data were generally consistent with those of the bioinformatics analyses. There was no evidence of systemic metastasis in any of these patients; therefore, no relationship between the expression of the 4 miRNAs and systemic metastasis of the tumor was identified.

4. Discussion

Dysregulation of miRNA expression has been described in a variety of diseases, particularly in cancers, including HNSCC. Increasing evidence has shown that miRNAs play an important role during HNSCC initiation and progression. It was reported that the overexpression of miR-96-5p led to increased cell migration and chemotherapy resistance in HNSCC cells. In addition, it was found that miR-30e-5p could repress angiogenesis and metastasis by directly targeting AEG-1 in squamous cell carcinoma of the head and neck. Although the roles of miRNAs in regulating the development and progression of HNSCC have been widely investigated, the underlying mechanisms are still unclear. Therefore, comprehensive studies are required to identify potential molecular therapeutic targets in HNSCC. In this study, we investigated the miRNA profiles of HNSCC tissues and adjacent control tissues available in the TCGA dataset. We identified 4 miRNAs that were aberrantly expressed in HNSCC and found that they were closely associated with disease progression in these patients. The expression of these 4 miRNAs was then verified in clinical HNSCC samples and paired adjacent control tissues through searches of the GEO database.

Three of the 4 miRNAs identified in this study have been implicated previously as effective molecular biomarkers of some

| miRNA name   | miRBase ID       | Location                        | log₂FC      | AUC (95% CI)   | P-value    |
|--------------|------------------|---------------------------------|-------------|----------------|------------|
| hsa-miR-410-3p | MIMAT0002171     | chr14:101065912-101065991 (+)  | -2.153883092| 0.8574 (0.8074-0.9073) | 4.23E-25   |
| hsa-miR-411-5p | MIMAT0003329     | chr14:101023325-101023420 (+)  | -2.279278371| 0.8687 (0.8178-0.9195) | 8.66E-31   |
| hsa-miR-125b-2-3p | MIMAT0004603 | chr21:16590237-16590325 (+)    | -2.057123274| 0.8687 (0.8254-0.9121) | 2.11E-18   |
| hsa-miR-99a-3p | MIMAT0004511     | chr21:16539089-16539169 (+)    | -1.734951148| 0.8409 (0.7933-0.8886) | 4.71E-16   |

AUC = area under the ROC curve, CI = confidence interval, miRNAs = microRNAs.

Figure 2. Clinical value of the 4 aberrantly expressed miRNAs in patients with HNSCC. ROC curves of the top 4 aberrantly expressed miRNAs sorted by AUC. Red and blue lines represent the sensitivity curve and identification line, respectively. The x-axis labeled “1-Specificity” indicates the false positive rate. The y-axis labeled “Sensitivity” indicates the true positive rate. Data were from TCGA database. AUC = area under the ROC curve, HNSCC = head and neck squamous cell carcinoma, miRNAs = microRNAs, ROC = receiver-operating characteristic, TCGA = the Cancer Genome Atlas.
special diseases. Zhang demonstrated that hsa-miR-411-5p was significantly downregulated in breast cancer.[22] Li reported that hsa-miR-125b-2-3p may be a potential biomarker of ischemic stroke.[23] Recently, Okada confirmed low expression of miR-99a-5p and miR-99a-3p significantly predicts poor prognosis in HNSCC, and these miRNAs regulate cancer cell migration and invasion.[24] Arai confirmed that hsa-miR-99a-3p acts as an antitumor miRNA in castration-resistant prostate cancer.[25] Wang proved that miR-410-3p functions as a tumor suppressor in glioma by directly targeting RAP1A.[26] However, reports describing the function of these 4 miRNAs in HNSCC are still rare.

Figure 3. Expression of the top 4 aberrantly expressed miRNAs between HNSCC and adjacent normal tissues. Data from TCGA dataset represent the mean ± SD, **P < .05, ***P < .001. HNSCC = head and neck squamous cell carcinoma, miRNAs = microRNAs, SD = standard deviation, TCGA = the Cancer Genome Atlas.

Figure 4. Association between the expression of the top 4 aberrantly expressed miRNAs and clinicopathological parameters of patients with HNSCC. Data from TCGA dataset represent the mean (range), **P < .05, *P < .01. HNSCC = head and neck squamous cell carcinoma, miRNAs = microRNAs, SD = standard deviation, TCGA = the Cancer Genome Atlas.
Figure 5. Validation of the expression of the top 4 aberrantly expressed miRNAs using the GEO database. (A) Expression of the 4 miRNAs in HNSCC and paired adjacent control tissues. (B) The expression of the 4 miRNAs in HNSCC and paired adjacent control tissues. Data from GEO dataset were analyzed using Student t test; \( P < .05 \). GEO = Gene Expression Omnibus, HNSCC = head and neck squamous cell carcinoma, miRNAs = microRNAs.
In the present study, the top 4 aberrantly expressed miRNAs (hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p) showed high diagnostic value for HNSCC, with AUCs exceeding 0.80. The findings based on TCGA database were then confirmed with independent data from the GEO database. Furthermore, examination of associated molecular pathways indicated the association of this 4-miRNA signature with regulation of transcription, cell proliferation, and apoptosis. The regulation of transcription affects genome instability, further increasing the possibility of carcinogenesis. The regulation of cell proliferation and apoptosis affects tumor occurrence and development.

By analyzing KEGG pathways, we found that differentially expressed miRNAs were mainly enriched in the TGF-β signaling pathway and the Hippo signaling pathway, which is critically involved in the regulating cell growth, differentiation, and development. Deregulated TGF-β expression has been reported to play important roles in tumor occurrence and progression in many types of cancer. Pang reported that TGF-β signaling promotes tumor progression in HNSCC via its effects on a variety of immune cells. Hui observed over-expression of miR-25, miR-93, and miR-106b regulated TGF-β signaling in HNSCC. Furthermore, KEGG pathway analysis revealed enrichment of the aberrantly expressed miRNAs in the Hippo signaling pathway. This evolutionarily conserved pathway controls organ size by regulating cell proliferation and apoptosis as well as self-renewal of stem cells. You has reported that miRNA-495 confers inhibitory effects on cancer stem cells in oral squamous cell carcinoma through the HOXC6-mediated TGF-β signaling pathway. Similar to the TGF-β signaling pathway, Hippo signaling pathway dysregulation has been shown to contribute to the development of cancer. Martin confirmed that FAT1 acts as a tumor suppressor in HNSCC cells by inhibiting the Hippo signaling pathway. Alzahrani revealed that Hippo signaling pathway dysregulation is involved in oropharyngeal squamous cell carcinoma tumorigenesis. In addition, the Hippo effector tafazzin can promote cancer stemness by transcriptional activation of sex determining region Y box 2 in head and neck squamous cell carcinoma. Therefore, it can be speculated that the 4 miRNAs detected in this study may affect tumorigenesis and progression in HNSCC by regulating the TGF-β and Hippo signaling pathways.

In this study, we observed significant downregulation of hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, and hsa-miR-99a-3p in HNSCC tissues samples compared with those in paired adjacent control tissues. In addition, high expression of these markers was associated with low TNM staging in patients with TCGA database and low TN staging in our clinical data. MiRNAs usually reduce or prevent protein synthesis by arresting gene translation at the post-transcriptional level. Therefore, we speculate that hsa-miR-410-3p, hsa-miR-411-5p, hsa-miR-125b-2-3p, hsa-miR-99a-3p have anticancer effects in HNSCC and their target genes may be potential oncogenes; however, the exact mechanism requires further investigation. All 22 patients included in this study required surgery and none had distant metastasis. It is possible that those patients with distant metastasis did not come to our center because they had no opportunity for surgery. Therefore, we were unable to further
Figure 8. Expression of the top 4 aberrantly expressed miRNAs between HNSCC and control samples. U6 was used as endogenous control to normalize miRNA expression. The data are expressed as the mean (range) of the 3 independent experiments with a duplicate each time. (A) Expression of the 4 miRNAs in HNSCC and control tissues. (B) Association between the expression of these 4 aberrantly expressed miRNAs and clinicopathological parameters of patients with HNSCC. Data were analyzed by paired Student t test, *P < .05, **P < .01 indicate significant differences versus control. HNSCC = head and neck squamous cell carcinoma, miRNAs = microRNAs.
verify the relationship between these 4 abnormally expressed miRNAs and HNSCC systemic metastasis. The cross-talk between miRNAs and genes is an emerging focus of research. Therefore, we performed PPI as a preliminary investigation of the regulation of genes co-expressed with the 4 miRNAs. The PPI results indicated an association of the top 4 aberrantly expressed miRNAs with regulation of gene expression and critical cell functions. The co-expression network of miRNAs remains to be confirmed in HNSCC, and intensive studies of the roles of aberrantly expressed miRNAs in the pathogenesis of HNSCC are still required.

5. Conclusions

We have identified 4 aberrantly expressed miRNAs associated with HNSCC. Expression levels of these aberrantly expressed miRNAs were validated in clinical HNSCC samples. Our findings indicate the clinical value of this novel molecular signature as a diagnostic biomarker in this disease. However, the mechanism underlying the roles of these miRNAs in HNSCC remains to be fully elucidated in further intensive studies.

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