Overexpression of miRNA 4451 is Associated With a Poor Survival of Patients With Hypopharyngeal Cancer After Surgery With Postoperative Radiotherapy

Abstract

Hypopharyngeal cancer (HC) is the most common subset of head and neck cancers. These tumors often have an aggressive clinical outcome characterized by local invasion and regional nodal metastasis. Upregulated miRNAs might be useful as biomarkers for prognosis and molecular targets for these tumors. We determined tumor expression of candidate miRNAs using microarray in 8 HC patients and validated in 372 HC patients. We also used paired tumorous and mucosal tissue to verify the miRNA expression. Log-rank test and Cox model were used to evaluate the survival; and Harrell's C-index was used to compare concordance of Cox models. Our results indicated 7 miRNAs aberrantly expressed in HC. Three of these candidate miRNAs (miRNA-4415, miRNA-200a, and miRNA-30b) were selected for further qRT-PCR validation and all of them were frequently found upregulated in HC tumors; with miR-4451 being the most differentially expressed. Moreover, high expression of miR-4451 was positively correlated with advanced tumor stage and increased mortality risk (HR: 1.6, 95% CI: 1.2–2.3; adjusted HR: 1.5, adjusted 95% CI: 1.1–2.1). Finally, significantly higher expression of miR-4451 in tumors compared to in fresh adjacent normal tissues indicates an oncogenic role of miR-4451 in this tumor. Upregulated miR-4451 in HC samples were frequently found and is significantly associated with advanced stage and poor survival of HC, which may indicate an association of this miRNA with the carcinogenesis process in this tumor site; and they could serve as a prognostic biomarker as well as help develop potential new targets for therapy.

Introduction

Among head and neck cancers, hypopharyngeal cancer (HC) is less common than tumors at other sites, such as larynx, oral cavity, and oropharynx. It accounts for about 2.8–4.0% of head and neck cancers [1]. The average incidence was about 0.7 from 2006 to 2010 in United States with a declining trend [2,3]. The incidence in Netherland and United Kingdom were 0.95 and 0.63 [4,5]. However, even with treatment options that include surgery,
chemotherapy, radiotherapy, and targeted drug therapy, the prognosis of HC is the worst in head and neck cancers. The 5-year overall survival rates was about 35% [3,4]. Because of the complicated structure of the upper aerodigestive tract and the high local recurrence and nodular metastasis rates, the TNM staging system did not accurately predict outcomes with HC; and only few molecules were identified as prognostic biomarkers [6,7]. Thus, more robust and accurate molecular biomarkers for predicting the outcome are needed.

MiRNAs are a group of widely-spread endogenous, single-strand, non-coding nucleic acids. They are short, with length of 18–25 nucleotides (nt), and modulate mRNA expression. Synthesized in the nucleus, the pri-miRNAs were targeted transporting to plasma where the mature miRNAs were generated by splicing the pre-miRNAs. Via base pairing with the complementary sequence in the 3′ untranslated region, these non-coding small RNAs can silence the mRNA by site-specific cleavage, enhanced mRNA degradation, or translational inhibition [8]. A study suggests that aberrant miRNA expression plays a role in tumor biogenesis, invasion, and metastasis [9]. Up-regulated miRNAs in tumors may function as oncogenes by inhibiting the tumor suppressor gene, whereas down-regulated miRNAs may negatively regulate oncogenes [10]. Along with lncRNA and circRNA, the miRNA participates in the network of gene expression regulation. More than 2300 miRNAs in miRBase 21 have been identified for human being. About 30–60% protein-coding genes are regulated by miRNAs [11]. A growing body of evidence has shown signatures of aberrant miRNA expression profiles can serve as useful biomarkers for cancer prognosis and diagnosis [12].

Formalin-fixed, paraffin-embedded (FFPE) tissues are archived clinical materials of great research value. Under certain conditions, they can be stored for decades. A previous study showed that miRNAs in FFPE tissues were easily degenerated with only 3% of mRNA detectable by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis [13]. However, miRNAs are more robust than mRNA in FFPE tissue. It was proved that profiles of miRNA expressions in FFPE tissues was correlated with those of the corresponding fresh tissues [14]. Both qRT-PCR and microarray techniques could produce reliable and reproducible miRNA expression measurement.

To date, while dozens of miRNAs were revealed aberrantly expressed in head and neck carcinoma, few large-scale studies to detect valuable prognostic miRNAs in HC have been performed. In this study, we used miRNA microarray and qRT-PCR to a large cohort of patients with HC to identify candidate miRNAs as biomarkers for cancer prognosis.

Materials and Methods

Study Patients and Tissue Samples

We reviewed all the patients with biopsy-proven HC enrolled in Department of Otolaryngology, Qilu Hospital of Shandong University between January 2012 and July 2016. The inclusion criteria were patients who received surgery with post-operative radiotherapy. The exclusion criteria included: (1) patients who were lost during retrospectively interview; (2) medical records unavailable; (3) tumors without enough RNA for study. All the patients signed consents when admitted to the hospital. Patient vita status (death or alive) was retrospectively obtained from the patients’ relatives by either phone or mail during the follow up. The survival time was defined as months from surgery to death of any reason. The epidemiological variables of patients, including age, sex, exposure to cigarettes and alcohol, were extracted from medical records. Two senior physicians determined TNM categories based on the surgical records and pathology reports using the American Joint Committee on Cancer Staging Manual, 8th edition. Finally a total of 390 patients with FFPE tissues and data available were included from the Departments of Otolaryngology and Pathology, Qilu Hospital of Shandong University. This study was approved by Medical Ethnical Committee of the Qilu Hospital (2017061).

Clinical Specimens

The FFPE tissue blocks of patients’ tumors were made by certified pathological technicians under standard procedures. The tumors were collected during surgery, formalin-fixed immediately and paraffin-embedded within 3 hours. All the blocks were preserved in professional conditions to avoid moisture and light. In this cohort, for each patient’s sample, FFPE slices at 10 μm were made, with the superficial 3 slices discarded in case that the RNA was degenerated. Two certified pathologists marked the tumor region in one slice with hematoxylin–eosin staining. Following the stained tumor as marker, we excluded the mucosal stroma outside the tumor core in other stainless slices. A total sample of 2–4 sections per patient were used, depending on the size of the primary tumor.

MiRNA Extraction and Quality Control

Following the manufacturer’s protocol, the total RNA from FFPE samples was extracted by RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion, Waltham, MA), using the Xylene method for deparaffinization. NanoDrop ND 3.0 spectrophotometer (NanoDrop Technologies Inc., Waltham, MA) was used to determine the quality of total RNA samples, including concentration and purity. Agilent 2100 Bioanalyzer (Agilent Technologies, City of Santa Clara, CA) was used to assess the RNA integrity number (RIN).

High-Throughput miRNA Expression Profiling

To screen out the different miRNA expression profiles by high-throughput microarray technique, we briefly divided the cohort by vita status, specifically, death vs. alive groups, and 4 cases from each group were chosen. The total RNA of 100 ng was labeled with Cy3 fluorescent label after dephosphorylation and denaturation. Purified samples were hybridized with Agilent Human miRNA microarray (Release 21.0, 8’600 K). The arrays were washed twice and scanned by the Agilent G2505C Microarray Scanner System. Image analysis was carried out using the Agilent Feature Extraction Software. We used the Agilent GeneSpring GX Software the correct the qualified signals to background and normalized them by quantile method. For every miRNA probe, fold change (FC) value was calculated (by a formula 2^ [average value of death group – average value of survival group]). If the average value for the death group was greater than that for the alive group, this miRNA expression was labeled with upregulation and vice versa. Criteria for selecting candidate differently expressed miRNA were: (1) FC of miRNA between the two groups ≥2 and p value ≤0.05 (2) signals of miRNA probe could be detected in all 8 samples.

qRT-PCR Validation and Selection of Endogenous Control

The total RNA of 100 ng was polyadenylated and reversely transcribed to cDNA using Qiagen miScript II RT Kit (Qiagen, Germany) in a 20μL volume. The cDNA was diluted 10x and assayed
in 10µl volume PCR reactions using miScript SYBR Green PCR kit and miScript Primer Assays (Qiagen, Germany) in accord with the manufacturer’s instructions. For each 96-well plate, a single target was amplified. Each sample was detected repeatedly in 3 wells. A positive control (Small Nucleolar RNA, C/D Box 6 [RNU 6] mimics) was set as inter-plate calibrator (IC) to minimize the systemic amplification bias between different PCR runs [15]. All PCR reactions were performed in an ABI 7900HT amplifier under relative quantification mode. The amplification curves were generated by SDS Software 2.4 (ABI, Waltham, MA). Original data was exported to Thermo Fisher Cloud (https://apps.thermofisher.com/apps/dashboard/) to be analyzed on-line. The Cq values were adjusted by IC, and the expressions of target miRNA were calculated as $2^{\Delta \Delta Cq}$, and $[\Delta Cq = Cq$ (candidate miRNA) - Cq (endogenous control)]

To determine the appropriate endogenous control, 5 candidate normalizers, RNU6B, RNU44, RNU48, RNU66, and miR-16 were used following the previously published articles [16–20]. The expression levels of these controls were measured in 30 samples by another qRT-PCR with the same PCR system, as previously described. The exported Cq data was analyzed using NormFinder [21]. The candidate control with the best stability value was selected as endogenous control of the current study.

Further Validation of the Candidate miRNA in Fresh Tissues

Another ten pairs of fresh tumor and adjacent normal mucosal tissues were collected and stored immediately in liquid nitrogen. The tumor tissues were collected at the center of tumor without necrosis, and the normal mucosal tissues were cut from the mucosa 1 cm from the tumor margin in the same patient. MirVana™ miRNA Isolation Kit (Thermo Fisher, Waltham, MA) was used to extract small RNA in accordance with the manufacture’s instruction. The quality control of RNA was the same as samples used for the FFPE tissues. The qRT-PCR above was used to quantify the expression level of candidate miRNA. The 2-sided paired Wilcoxon test was used to determine whether there were significant differences ($P < .05$) between normal and tumorous tissues. Fold change was calculated as $2^{\Delta \Delta Cq}$.

Statistical Analysis

Statistical product and service solutions (Version 23; IBM, Waltham, MA) was used for analysis. Statistical significance was set at $P < .05$, and all tests were set at 2-sided. Chi-square tests were used to evaluate the differences between epidemiological/clinical conditions and the miRNA expressions. The Kaplan–Meier method was used to determine whether there were significant differences ($P < .05$) in survival between the two groups with epidemiological, clinical, and pathological conditions, as well as miRNA expressions. The end outcome of the study was set as overall death. Time to event was calculated as number of months from the date of surgery to occurrence of the event. The epidemiological variables included age, sex, and smoking and alcohol status, and the clinical characteristics consisted of the primary sites and tumor differentiation, TNM stage, and clinical stage. All the variables were transformed to categorical variables.

To build the multivariate model, the univariate analysis was performed for each variable with survival predictive potential suggested by the Kaplan Meier survival analysis. A forward stepwise (likelihood ratio) strategy was used and the cutoff threshold level for the likelihood ratio test was set at 0.25. Associations were quantified using hazard ratios (HRs) and their 95% confidence intervals (CIs) for survival. The fitness of multivariate Cox models was evaluated using the Harrel’s concordance index (C-index) to testify the predictive accuracy for each different model [22], as performed by RStudio (version 3.2.2).

Results

Identification of miR-4451 as a Significant Candidate miRNA for Study

We used the hybrid microRNA microarray consisted of 2549 mature miRNA probes to identify the candidate miRNAs for study. Eight FFPE samples from 4 patients in the alive group and 4 patients in the death group were used. Under the two screening conditions ($P < .05$ and FC [absolute value] > =2), a total of 7 differently expressed miRNAs were selected (miR-4451, miR-200c, miR-3161, miR-3605-5p, miR-378b, miR-429, and miR-4701). Among them, miR-200c, miR-429, and miR-4701 were down-regulated, whereas miR-3161, miR-3605-5p, miR-378b, and miR-4451 were up-regulated in the death group compared with those in the alive group. Furthermore, miR-4451 was found to have the highest FC and significance in expression difference (FC = 9.6; $P = .0053$). Thus, we selected miR-4451 as the candidate miRNA for further study for the prognosis, and the whole set of raw data can be accessed in GEO database (GSE117558).

Validation of miR-4451 Expression in 372 HC Patients by the RT-PCR

For the validation, a total of 436 patients with biopsy-proven HC cancer and medical records available; and there were 408 patients who received surgery and post-operative radiotherapy. Among them, 11 patients were lost for follow up and 8 patients with misclassification of diagnosis and tumor sites. In the remaining 390 patients with FFPE tissues available, 8 cases had less RNA amount for microarray analysis. Thus, all these patients were excluded; and a total of 372 patients were included for the final analysis.

To quantify the expression levels of miR-4451 in 372 patients, the total RNA by the Nanodrop and Agilent 2100 showed an average A260/280 ratio of 1.9 (SD = 0.084) and an average RIN of 2.3 (SD = 0.63). To identify the most stable endogenous control, we quantified five candidate endogenous controls; and RNU48, with the best stability value of 0.663, was selected as the endogenous reference using the NormFinder compared with the traditional reference gene RNU6 (stability value = 1.6) and miR-16 (stability value = 3.6). After correction for inter-plate deviation by positive control, the expression level of miR-4451 was calculated as $2^{\Delta \Delta Cq}$. The expression of miR-4451 in this cohort of 372 patients remained significantly higher in the death groups than in the alive groups ($P < .001$), with an approximately FC = 8.3.

Further Verification of miR-4451 Expression in Fresh HC and Adjacent Normal Tissues

To detect if miR-4451 expression is different between HC and adjacent normal tissues, we used the same methods to quantify miR-4451 expression in 10 paired patients in 10 patients with HC and 10 normal adjacent tissues. Our results showed that miR-4451 expression levels were significantly different between tumor and normal adjacent tissues ($P = .007$, Figure 1). The miR-4451 expression level in tumor tissue was approximately 2.1-fold higher than in that in normal adjacent tissues (0.017 vs 0.008), indicating miR-4451 was up-regulated in HC tumors.

Patient’s Characteristics and miR-4451 Expression in this Cohort
The epidemiological, clinical, and pathological variables, along with miR-4451 expression levels for this cohort of 372 patients are summarized in Table 1. The median age of patients at time of diagnosis was 59 years (mean: 59.3; SD: 8.6; range: 36–82); we used median age to divide the cohort into 2 age groups. In this cohort, 349 were male patients (93.8%), and 23 were female patients (6.2%). After follow up, we found that 149 patients (40.1%) died due to any causes. There was no significant association of survival with age and sex. Although smokers and alcohol users were predominant in this cohort, smoking and alcohol status were not associated with survival (All Log-rank, $P > .05$). However, the significant associations were found between survival and T, N, and overall stage (All log-rank, $P < .05$).

Table 2 shows the distribution of patients’ characteristics and miR-4451 expression. We did not find any significant differences of miR-4451 expression in age, sex, smoking, alcohol users, primary tumor sites, differentiation and N classification (All $P > .05$), while the significant differences were found only for T and overall disease stage ($P = .029$ for T classification and $P = .050$ for overall stage). The patients with T4 disease expressed 2.2-fold higher level of miR-4451 than the patients with T1–3 disease. Lymph node metastasis is an important prognostic factor in HC; however, miR-4451 expression was not associated with lymph node metastasis ($P = .186$).

Effect of miR-4451 Expression on Survival of HC Patients

The patients were divided into two groups based on the median of miR-4451 expression, with 182 in the low expression group and 190 in the high expression group. In the low expression group, 63 of 182 patients died during follow-up (34.6%), whereas in high expression group 86 of 190 patients died (45.3%). The Kaplan–Meier survival analysis showed that patient with high miR-4451 expression experienced a worse survival than patient with low miR-4451 expression (log-rank test, $P = .003$, Figure 2). In the univariate analysis, the high miR-4451 expressed patients had an approximately 1.6-fold significantly elevated risk for mortality, compared with the patients with...

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**Table 1.** Distribution of the Characteristics in the Patient Cohort

| Variables             | Total No. | Total % | $P$  |
|-----------------------|-----------|---------|------|
| Total patients        | 372       | 100     |      |
| Age, years            |           |         | .636 |
| ≤ Median of 59 years  | 177       | 47.6    |      |
| > Median of 59 years  | 195       | 52.4    |      |
| Sex                   |           |         | .880 |
| Male                  | 349       | 93.8    |      |
| Female                | 23        | 6.2     |      |
| Smoking Status        |           |         | .331 |
| Never                 | 73        | 19.6    |      |
| Ever                  | 299       | 80.4    |      |
| Alcohol               |           |         | .394 |
| Never                 | 96        | 25.8    |      |
| Ever                  | 276       | 74.2    |      |
| Primary Site          |           |         | .145 |
| Piriform              | 298       | 80.1    |      |
| Post Wall/Postcricoid | 74        | 19.9    |      |
| Differentiation       |           |         | .093 |
| Poor or Moderate      | 275       | 73.9    |      |
| Well                  | 97        | 26.1    |      |
| T classification      |           |         | .002*|
| T1/T2/T3             | 325       | 87.4    |      |
| T4                    | 47        | 12.6    |      |
| N classification      |           |         | .017*|
| N0/N1                 | 173       | 46.5    |      |
| N2/N3                 | 199       | 53.5    |      |
| Clinical Stage        |           |         | .006*|
| I/ II /III            | 153       | 41.1    |      |
| IV                    | 219       | 58.9    |      |

*P: Log-rank test was used for differences between different subgroups. $P < .05$. $P ≤ .05$. $P < .05$.
low miR-4451 expression (adjusted HR, 1.5; 95% CI, 1.1–2.1) as shown in Table 3.

To testify the statistical concordance, we used Harrel’s C-index to quantify the predictive accuracy of the multivariate models (Table 4). For a multivariate Cox model with T classification, N classification as independent variables, the C-index was 0.58. When adding overall stage, the value slightly elevated to 0.59 but did not reach statistical significance (P = .353). However, the C-index rose to 0.61 when adding miR-4451 expression to variables, and the statistical difference between the two models was significant (P = .040). Finally, when we added the miR-4451 expression and primary site both to the model, the C-index rose to 0.62 (95% CI: 0.57–0.66) and the concordance of the model was significantly higher than the original model that included only T and N classification (P = .018).

**Discussion**

In this analysis, we used miRNA microarray to identify miR-4451 as a potential independent prognostic factor and further verified this finding in a larger patient cohort. Overexpression of miR-4451 increased the risk of mortality for patients with HC. In addition, miR-4451 expressed approximately 2-fold higher in HC tumor tissues than in normal adjacent tissues, indicating that miR-4451 may play an oncogenic role in HC development.

HC has the worst prognosis among head and neck squamous cell carcinomas. Its location is occult and would often be omitted during the routine health examination. Unlike laryngeal cancer with early hoarseness and oropharyngeal cancer which can be directly visualized, the symptoms of early HC are non-specific. Patients usually were treated for chronic pharyngitis until late stage with dysphagia or dyspnea. Our study patients showed that the distribution of the cohort was inclined toward phase III and IV of clinical stage. Therefore, compared with the minimal invasive procedures for laryngeal or oropharyngeal carcinoma, the mainstream for managing HC is still open surgery and chemoradiotherapy.\[23,24\]. However, an important characteristic of the hypopharyngeal region is that there is no intensive barrier, and thus the mucous invasion of the tumor was prominent. Adjacent structures, such as the larynx or esophagus, are often involved. Even for resectable HC tumors, satellite metastases could remain after the surgical procedure, which needs adjuvant post- or pre-operative chemoradiotherapy to control local recurrence.

Another negative factor for natural history of HC is the abundant lymphatic drainage, which leads to a high risk of nodal metastasis and regional recurrence. These two characteristics are reflected as T

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**Table 3.** Association of miR-4451 Expression With OS of Hypopharyngeal Carcinoma Patients (N = 372) *

| Expression | Death/Total | Survival | Univariate Analysis | Multivariate Analysis |
|------------|-------------|----------|---------------------|-----------------------|
|            | No. | %     | HR   | 95% CI | P    | HR   | 95% CI | P    |
| miR-4451   | Low  | 63/182 | 34.6 | 1.0   | .003 | 1.00 | .023  |
|            | High | 86/190 | 45.3 | 1.6   | 1.2–2.3 | 1.5 | 1.1–2.1 |

* P ≤ .05.

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**Table 4.** Harrel’s C-index for Different Multivariate Cox Model

| Variables included in Multivariate Cox Model | c-index | 95%CI | P |
|---------------------------------------------|---------|------|---|
| T classification N classification            | 0.58    | 0.58 | 0.62 | reference |
| T classification N classification Overall stage | 0.59 | 0.55 | 0.64 | 0.353 |
| T classification N classification miR-4451   | 0.61    | 0.56 | 0.65 | 0.040* |
| T classification N classification miR-4451 Overall stage | 0.62 | 0.57 | 0.66 | 0.018* |

* P ≤ .05.
classification and N classification; therefore, the TNM staging system is an important prognostic method for HC [25]. Our current research also support that the T and N classifications were associated with survival of HC; and high grades of T and N state increase the mortality. Although the TNM staging system has been a well-accepted method to forecast prognosis of HC, many studies have made effort to find tissue or serum biomarkers to enhance the prognostic accuracy. As an important prognostic factor in head and neck carcinoma, human papillomavirus (HPV) status seems to be prevalent in oropharyngeal carcinoma. Dahm showed that HPV infection did exist in HC patients; however, there was no evidence for a better survival for HPV-positive patients with HC [26].

So far, several molecules were found to be biomarkers for prognosis of HC. As major transcription factor associated with stem cell self-renewal and differentiation, high expression of Oct 4 in HC is correlated with worse prognosis. Co-expression of Sox2 further elevates the risk of mortality [6]. Elevated osteopontin, which functioned as a cell attachment protein, in plasma or tumor tissues of HC, was also a useful indicator for prognosis [27]. Omura showed that truncating mutations of TP53 mutation status was correlated with poor prognosis in surgically treated patients [7].

Besides the protein, miRNA as newly discovered non-coding RNA has been evaluated as a potential biomarker. These small RNAs are quite stable and easily detected, so both FFPE and blood samples can be used for clinical evaluation. Various miRNAs have been found aberrantly expressed. Some were down-regulated such as miR-517c, miR-196a, miR-7, miR-21, and et al. [28,29], whereas some were up-regulated such as miR-1, miR-375, miR-139-5p, miR-504, and et al. [28,30]. Few studies have been focused roles of miRNAs in HC. Recently, Kikkawa et al. found that miR-504 inhibited cancer cell proliferation via targeting CDK6 in hypopharyngeal squamous cell carcinoma [30].

In this study, despite the unknown mechanism, we focused on miR-4451 as a novel prognostic factor in HC. Mao et al. verified that miR-4451 was down-regulated in pancreatic ductal adenocarcinoma [31], while we found that miR-4451 might be an oncogenic miRNA that is associated with worse prognosis in the current study, which is further supported by our validation in our fresh tissues using RNU48 as the reliable endogenous control for miRNA quantification [33,34].

Our study has several strengths. Firstly, this is a relatively large cohort of patients with HC, low rate of lost for follow up; secondly, all the data and miRNA quantitation were carefully documented; thirdly, the association of miR-4451 with survival was fully adjusted with several important prognostic confounders; and finally, The multivariate Cox regression models were further evaluated for the concordance. Nevertheless, even though these advantages, our study still has some limitations. First, this study is a retrospective analysis, while a recall bias might exist. Second, in our cohort, the patients were from a single hospital, potential confounders could not avoided due to the hospital-based cohort. Thus, multicenter analysis is needed. Finally, the exact mechanism behind this association of miR-4451 with prognosis remains unclear, further oncogenic roles of this miRNA should be investigated requires more in vivo and in vitro experiments.

In conclusion, expression of miR-4451 is correlated with progression and worse survival of HC patients; and might serve as a prognostic biomarker for HC. Moreover, miR-4451 may play important oncogenic role in development of HC. The upregulated of this miRNA in HC tissues, which could indicate an association of this gene with the carcinogenesis process in this specific tumor site and it can help develop potential new targets for future therapy.

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