Original Article
Downregulation of serum miR-329 is an unfavorable prognostic biomarker in head and neck squamous cell carcinoma

Dongying Li1,2, Zezhang Tao1
1Department of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, China; 2Department of Otolaryngology Head and Neck Surgery, Baoan Central Hospital of Shenzhen, Shenzhen, China

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Abstract: MicroRNAs (miRNAs) have been shown to play a central role in regulating many important biological processes. Aberrant expression of miRNAs is closely associated with cancer development. The goal of this study was to determine the clinical significance of serum miR-329 in head and neck squamous cell carcinoma (HNSC). Real-time PCR was used to examine serum miR-329 levels in HNSC patients and healthy volunteers. Then the diagnosis and prognostic value of serum miR-329 was investigated. Our results showed that serum miR-329 level was significantly downregulated in patients with HNSC compared to the healthy controls. In addition, serum miR-329 not only could differentiate HNSC patients from healthy controls, but also discriminated the HNSC patients at different clinical stages and with different lymph node metastasis status. Low serum miR-329 level was significantly associated with clinical stage and lymph node metastasis. Moreover, patients in the low serum miR-329 group had poorer 5 year overall and recurrence free survival than those in the high serum miR-329 group. Finally serum miR-329 was an independent prognostic risk factor for HNSC. Collectively, the current study was the first to evaluate the clinical value of serum miR-329 in HNSC. These findings suggest that serum miR-329 is a potential diagnostic and prognostic biomarker for HNSC.

Keywords: Biomarker, diagnostic, HNSC, prognostic, serum miR-329

Introduction

Head and neck cancer is the sixth most common cancer around the world and represents a significant public health issue [1]. Head and neck squamous cell carcinoma (HNSC) is the most common subtype accounting for about 85% of all cases [2]. Most HNSC patients are diagnosed at advanced stage, which leads to the poor clinical outcome of this deadly disease [3, 4]. Currently tumor, node, metastasis (TNM) staging is a commonly used prognostic index for HNSC, but it is not sensitive enough. Therefore, it is important to explore novel prognostic marker to improve treatment stratification and overall survival.

MicroRNAs (miRNAs) are a class of small regulatory RNAs that regulate gene expression at the post-transcriptional level [5]. The regulation effects can be either translational inhibition or transcriptional silencing [6]. Overwhelming reports have demonstrated miRNAs are crucial for the initiation and progression of many types of cancer including HNSC [7]. The expression level of miR-203 was reduced during the epithelial-mesenchymal transition induction. Ectopic expression of miR-203 inhibited invasion and induced mesenchymal-epithelial transition in head and neck cancer cells. In addition, NUAK family SNF1-like kinase 1 was identified as a downstream target of miR-203, indicating miR-203 might play a tumor suppressive role in HNSC [8].

Reduced miR-146a and miR-155 levels in clinical samples were associated with poor clinical outcome of HNSC. Moreover, knock-down of either miR-146a or miR-155 promoted the proliferation and invasion capacity of head and neck cancer cells, and inhibitory effects were
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Aberrant expression of miR-329 has been reported in several types of cancers [10-14]. However, its role in HNSC remains poorly known. It is impossible to monitor the disease status as well as the therapeutic responses in real time using the tissue biomarkers. The molecules in the biofluid samples are good candidates to solve this problem. Therefore the goal of this study was to elucidate the expression level of serum miR-329 and its potential clinical significance in HNSC.

**Materials and methods**

**Ethics statement**

Serum samples and medical records were obtained from a cohort of 124 individuals at Renmin Hospital of Wuhan University. Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of our hospital, conforming to the ethical guidelines of Helsinki Declaration.

**Patients**

The study included 94 histopathologically confirmed, newly diagnosed untreated patients with HNSC and 30 healthy volunteers. HNSC patients were staged according to the 7th edition of UICC TNM classification. None of the HNSC patients had received chemotherapy or radiotherapy prior to serum collection. Five milliliters of peripheral blood samples were obtained from all participants within 6 h after collection. Serum was separated from the blood samples by centrifugation at 1200 g for 20 min and then stored at -80°C until further use. The baseline characteristics of HNSC cases were summarized in Table 1.

**Real-time PCR**

miRVana PARIS Kit (Ambion, Austin, TX, USA) was used to extract the RNA from serum samples according to the manufacturer's instructions. RNA was converted to complementary DNA with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Foster City, CA, USA). Amplification of cDNA was using SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan). The miRNA expression levels were measured on a 7500 Real-Time PCR system (Applied Biosystems). Relative expression of miR-329 was calculated with 2-ΔCt method and adjusted by U6 snRNA as an internal control.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0 and SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). A P-value of <0.05 was considered statistically significant. Mann-Whitney test was used for comparing the serum miR-329 levels between HNSC patients and healthy volunteers. Chi-square test was used for the categorical comparisons. The prediction value of serum miR-329 was analyzed by receiver operating characteristic (ROC) curve. Survival analyses were performed by the Kaplan-Meier method and log-rank tests. Univariate and multivariate analyses were used

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**Table 1. Serum miR-329 levels and clinicopathological parameters of HNSC**

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Serum miR-329 expression</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Low (44)</td>
<td>High (50)</td>
</tr>
<tr>
<td>Age</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>48</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>≥60</td>
<td>46</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Gender</td>
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<td>29</td>
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</tr>
<tr>
<td>Female</td>
<td>31</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>52</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td>T3-T4</td>
<td>42</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>39</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
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<td>17</td>
<td>38</td>
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<tr>
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<td>Negative</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>I-II</td>
<td>43</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>III-IV</td>
<td>51</td>
<td>33</td>
<td>18</td>
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<tr>
<td>Histological grade</td>
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<tr>
<td>Well + Moderate</td>
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<td>26</td>
<td>35</td>
</tr>
<tr>
<td>Poor</td>
<td>33</td>
<td>18</td>
<td>15</td>
</tr>
</tbody>
</table>
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Results

Serum miR-329 was reduced in patients with HNSC

Our real-time PCR result demonstrated the expression level of miR-329 was significantly decreased in the serum samples from HNSC patients compared to those from healthy volunteers (P<0.01) (Figure 1A). In addition, the ROC analysis showed that serum miR-329 had a good diagnostic value with an area under the curve (AUC) of 0.883 (specificity =0.90, sensitivity =0.78) (Figure 1B).

We then compared the serum miR-329 levels in HNSC patients at different clinical stages or different lymph node metastasis status. The results showed that HNSC patients at advanced clinical stage (III-IV) had remarkably lower serum miR-329 levels in comparison with those at the early clinical stage (I-II) (P<0.01). Moreover, Serum miR-329 level was able to discriminate HNSC at different clinical stages (AUC=0.783, specificity =0.85, sensitivity =0.74). The expression level of serum miR-329 was significantly decreased in HNSC patients with positive lymph node metastasis compared with those without lymph node metastasis (P<0.01). It also had good discriminative ability for lymph node metastasis status (AUC=0.747, specificity =0.82, sensitivity =0.72) (Figure 2).

Correlation of clinicopathological parameters with serum miR-329 in HNSC

The Chi-squared analysis showed that low serum miR-329 levels was significantly associated with advanced clinical stage (P<0.001) and positive lymph node metastasis (P<0.001). However, it was not correlated with age, gender, T stage, distant metastasis and histological grade (Table 1).

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Survival analysis showed that HNSC patients in the low serum miR-329 group had a significantly shorter 5 year overall survival time than the patients in high serum miR-329 group (P=0.003) (Figure 3).

We then compared the serum miR-329 levels in 45 HNSC patients who suffered from disease recurrence. Our results showed that serum miR-329 levels were significantly lower in the recurrent samples compared to the samples taken before any treatment (P<0.01) (Figure 4A). In addition, HNSC patients with lower serum miR-329 levels suffered worse 5 year recurrence free survival (P=0.0068) (Figure 4B).

Multivariate analysis showed that lymph node metastasis (HR=3.62, 95% CI=1.28-6.23, P=0.012), clinical stage (HR=5.40, 95% CI=1.71-11.39, P<0.001) and serum miR-329 level (HR=3.81, 95% CI=1.34-7.12, P=0.007) were
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Despite great advances in the past few decades, the five-year overall survival of HNSC remains stagnant. Late diagnosis is one of the major reasons for the high mortality rate of HNSC. MiRNAs play a central role in regulating a number of biological processes and deregulation of miRNAs is involved in the progression of many human diseases [15]. MiRNAs have been regarded as important players in cancer development. In addition, miRNAs are highly stable in biofluids such as serum, plasma, urine, and saliva [16]. Therefore, circulating miRNAs secreted from cancer cells might be

Discussion

Figure 2. The diagnostic value of serum miR-329 in HNSC patients with different clinical stages and lymph node metastasis.

Figure 3. The association between serum miR-329 levels and overall survival.

independent prognostic factors for HNSC (Table 2).
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In this study, we demonstrated that serum miR-329 was downregulated in HNSC and it could distinguish the HNSC patients from healthy people effectively. In addition, it was able to discriminate the HNSC patients at different clinical stages and with different lymph node metastasis status. Low serum miR-329 was positively correlated with advanced clinical stage and lymph node metastasis as well as poor survival. Moreover, serum miR-329 was an independent prognostic factor for HNSC, indicating serum miR-329 was a promising marker for patient’s stratification and therapeutic responses evaluation in HNSC. One limitation of current study was that the sample size was relatively small. Future studies with larger sample size and longer follow-up time should be performed to validate our findings. In addition, the correlation between serum miR-329 and tissue miR-329 needed to be further examined, as combination of these two markers might have better performance in the predicting the prognosis of HNSC.

In addition to head and neck cancer, miR-329 has been reported to function as a tumor suppressor in many types of cancers. The expression level of miR-329 was remarkably decreased in metastatic neuroblastoma in comparison with matched primary tumor. In addition, ectopic expression of miR-329 inhibited the proliferation, migration and invasion of neuroblastoma cells, and opposite findings were observed when miR-329 was inhibited. Furthermore, lysine-specific demethylase 1 was a downstream target of miR-329 [17]. Similarly, miR-329 was decreased in breast cancer and it could suppress the oncogenic behaviors of breast cancer cells both in vitro and in vivo by downregulating p130Cas, indicating that miR-329 acted as a tumor suppressor in breast cancer [18]. miR-329 was commonly decreased in gastric cancer and associated with advanced stage. Upregulation of miR-329 inhibited the proliferation, migration and invasion capacity of gastric cancer cells and T lymphoma invasion and metastasis 1 was a direct target of miR-329 [19]. To the best of our knowledge, no study has reported the oncogenic role of miR-329 in cancer so far, suggesting that the molecular function of miR-329 is independent from the tumor type and microenvironment. Further studies should elucidate the molecular mechanisms that accounting for the tumor suppressive role of miR-329 in cancer.

Collectively, the current study demonstrated that serum miRNA-329 expression level was decreased in HNSC patients, and associated with poor prognosis of this malignancy. Therefore, serum miR-329 is a novel and promising prognostic biomarker for HNSC, which has great potential to improve personalized therapeutic strategies.

Table 2. Multivariate analyses of different prognostic parameters for HNSC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
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<tbody>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>3.62</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>5.40</td>
</tr>
<tr>
<td>Serum miR-329 expression level</td>
<td>3.81</td>
</tr>
</tbody>
</table>

HR, Hazard ratio; CI, confidence interval.
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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zezhang Tao, Department of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, China. Tel: +86-27-8804-1911-86605; Fax: +86-27-8804-3958; E-mail: zezhangtaowhu@163.com

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