Original Article

Expression of ZO-1 in laryngeal squamous cell carcinoma and its prognostic value

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Abstract: Laryngeal cancer is one of the most common fatal cancers in head and neck carcinomas, but its mechanism is still unclear. The zonula occludens-1 (ZO-1) is closely related to the genesis and development of a variety of respiratory tract and gastrointestinal tumors but the expression of ZO-1 in laryngeal squamous cell carcinoma (LSCC) and its correlation with clinicopathologic features still remain undetermined. The aim to this study is to identify the clinical significance of ZO-1 in laryngeal cancer. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry with tissue microarrays were used to characterize the expression of the ZO-1 mRNA and protein in LSCC. The statistical analysis was carried out by combining the follow-up data and clinicopathologic features. Both the ZO-1 mRNA and protein expressions in LSCC are lower than their expressions in corresponding peritumoral tissue (P<0.05); the ZO-1 expression is related to tumor differentiation (P=0.009), clinicopathological stages (P=0.025) and lymphatic metastasis (P=0.013). As shown in the Cox regression analyses, the ZO-1 expression (P=0.035), tumor location (P=0.030), lymphatic metastasis (P=0.002) and tumor differentiation (P=0.004) are independent prognostic factors. The ZO-1 can serve as an independent prognostic factor of LSCC and the high expression of ZO-1 is associated with good prognosis.

Keywords: ZO-1, laryngeal neoplasms, squamous cell carcinoma, tumor marker

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common malignant tumor of head and neck and its incidence in primary respiratory cancer ranks only second to lung cancer at about 1/4 of global annual head and neck squamous cell carcinomas [1, 2]. Tumor stage, pathologic differentiation, primary site, and lymph node metastasis of LSCC are correlated with prognosis. These indexes, however, are insufficient for precise assessment of the tumor. Therefore, a thorough investigation of potential molecular biomarkers in predicting laryngeal SCC prognosis is of great significance [3, 4].

Tight junctions (TJs) are a sort of top connexin which can mediate the adhesion between epithelial cells and endothelial cells by combining with other cellular connexins [5]. Previous studies have reported that TJs can serve as barriers for tight junctions of multiple tissues and indexes of permeability function and participate in the maintaining and adjusting the epithelial barrier function such as blood brain barrier, enteroocyte and retinal pigment epithelium etc [6, 7]. They have many types including the Occludin, Claudins, Junctional Adhesion Molecule-A (JAM-A) and ZO family etc. Among them, the ZO family is the important factor of epithelial cell structure and function regulation mainly including ZO-1, ZO-2 and ZO-3 [8].

Zonula Occludens-1 (ZO-1) protein, which belongs to the membrane-associated guanylic acid protein and is the first tight junction protein found in epithelial cells. It is not only the major structural protein but also functional protein of tight junction, plays an important role in regulating the permeability of epithelial cell and main-
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Retaining cell polarity, and, meanwhile, has the characteristics of epithelial marker [9]. Researches show that decreased expression of ZO-1 in lung cancer and colon cancer tissues can facilitate the occurrence of lung cancer and colon cancer. The decrease in ZO-1 mRNA may be one of the indicators of early development of lung cancer [10, 11].

The expression of ZO-1 in LSCC and its relationship with the clinicopathologic features of LSCC are remain unknown. We used the qRT-PCR to detect the ZO-1 mRNA expression in 18 pairs of fresh LSCC tissues and its corresponding peritumoral tissue. In addition, we prepared tissue microarrays of LSCC, and determined the expression of ZO-1 in LSCC and corresponding adjacent tissues using immunohistochemistry, and analyzed its relationship with clinicopathologic parameters of patients with LSCC.

Materials and methods

Clinical information and preparation of tissue microarrays

All hospitalized patients between January 2002 and December 2010 were enrolled from the Department of Otolaryngology/Head and Neck Surgery, Nantong University. All patients were diagnosed with laryngeal SCC in accord with the World Health Organization (WHO) criteria [12] and TNM (tumor, node, metastasis) classification (Union Internationale Contre le Cancer [UICC]) and underwent total or partial laryngectomy and neck dissection (unilateral or bilateral, radical or functional, based on clinical and surgical findings). Nodal metastasis was confirmed by postoperative histologic examination. In this study, 98 paraffin-fixed tissue samples of LSCC and 26 control samples were prepared as tissue microarrays. Another 18 fresh LSCCs and surrounding normal tissues were collected as controls. None of the patients had received preoperative radiotherapy or chemotherapy. Complete follow-up data until December 2015 were documented in all these cases.

Tissue microarrays were produced by Shanghai Xinchao Bio-tech Co. Ltd. The representative cancer area was labeled in specific paraffin blocks in accord with hematoxylin and eosin staining results. A tissue array needle was inserted to obtain a 2-mm-diameter tissue sample, with 1 core for each sample. The tissue was then sequentially aligned into the preprepared blank paraffin blocks. The tissue microarrays were cut into 4-μm sections and placed on tissue microarray-specific adhesive-coated glass slides. Ethical approval to perform this study was obtained from the Human Research Ethics Committee of the local hospital.

One-step quantitative polymerase chain reaction

The primers of ZO-1 (NC_000015) and endogenous control GAPDH (NM_002046) were designed with the software Primer Premier 5.0. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services. Total RNA was extracted from 18 laryngeal SCC samples and their adjacent peritumoral tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA). Expressions of ZO-1 and GAPDH were determined by real-time PCR with IQ5 (Bio-Rad, Hercules, CA) using SensiMix One-Step Kit-based SYBR Green method (Quantace, London, UK). The primers for ZO-1 were as follows: forward primer 5'-GTTGGATCACTTGT-3' and reverse primer 5'-GATGATGCCTCGTTCTAC-3' for GAPDH, forward primer 5'-TCGGAGTCAACGGATTTGGTCGT-3', reverse primer 5'-TGCCATGGGTGGAATCATATTGGA-3'. Amplification conditions consisted of 30 minutes at 42°C for reverse transcription and 2 minutes at 94°C for Taq activation, followed by 35 cycles of 95°C for 20 seconds, 56°C for 20 seconds, and elongation at 72°C for 30 seconds. Each measurement was performed in triplicate.

Immunohistochemical staining and scoring

As for the aforesaid tissue microarrays, the two-step immunohistochemical method as well as the method of SP-9000 kit (Zymed Laboratories, USA) were adopted. After being preheated in an oven with a temperature of 60°C for six hours, tissue microarrays were dewaxed with gradient ethyl alcohol, hydrated with xylene and antigenically restored under high pressure with citrate solution (pH=6.0); after cooling to the indoor temperature, tissue microarrays were washed with phosphate buffered saline (PBS) every ten minutes for three times; the ZO-1 antibody (1:100) (Zymed Laboratories, USA) was dropped; tissue chips incubated over-
night at the temperature of 4°C and were washed with PBS every ten minutes for three times; secondary antibodies (1:50) were dropped; color development of diaminobenidine (DAB) (Dako, Germany) was controlled under an optical microscope; after being re-dyed with hematoxylin, they were dehydrated with gradient alcohol and hyalinized with hyalinize. PBS instead of primary antibodies was used as the negative control.

The double blind method was used to judge the immunohistochemical result; two pathological doctors judged the staining result under the optical microscope. Discordant cases were reevaluated under a double-headed microscope to achieve a consensus. In the case of disagreement, the slides were reviewed by a third pathologist until a consensus score was established. The rate of positive cells and staining strength were scored respectively; the percentage of ZO-1 positive cells was scored as follows: 0 for 0-19%, 1 for 20%-39%, 2 for 40-59%, 3 for 60-100%. Staining strength was scored as follows: 0 for no staining; 1 for weak staining and light yellow; 2 for medium staining and brown; 3 for strong staining and dark brown. The product of the percentage of positive cells and scores of staining strength is the final score, namely the staining index which was defined as follows: 6-9 scores: high expression; 0-4 scores: low expression [13].

Statistical analysis

Statistical analysis was carried out with SPSS19.0 software. The paired t-test was taken for the expression of ZO-1 mRNA in LSCC and peritumoral tissue; the chi-square test was taken for the ZO-1 protein expression in LSCC tissues and peritumoral tissue. The Mann-Whitney U test in the rank sum test was conducted to analyze the relation between ZO-1 and all clinicopathological parameters; Survival curves were calculated using the Kaplan-Meier method, and the Log-rank test was used for analysis; Multivariate analysis was performed using Cox proportional hazards model, the risk ratio and its 95% confidence interval were recorded for each marker; P values<0.05 were considered statistically significant.

Results

Patient demographics and tumor clinicopathologic characteristics

Among 98 patients (96 men and 2 women; mean age, 58.4 years; age range, 29-84 years), 28 patients (28.6%) presented with supraglottic laryngeal SCC and 70 (71.4%) with glottic laryngeal SCC. Seventeen cases (17.3%) were complicated with lymph node metastasis. Regarding the histologic differentiation, there were 48 patients (49.0%) with well-differentiated, 50 (51.0%) with moderately differentiated and poorly differentiated. In terms of distribution of TNM classification, 62 patients (63.3%) were found with stage I-II, 36 (33.7%) with stage III-IV tumors. Patients were divided into 2 groups by the total exposure (pack-years) to cigarette smoking [14], 31 patients (31.6%) with <60 pack-years were grouped together and the other group consisted of 67 patients (68.4%) with >60 pack-years. The 5-year overall survival was calculated from the date of surgery until the date of death or last follow-up. Five-year overall survival is 70.4% (69/98).

Results of quantitative polymerase chain reaction

qRT-PCR was used to detected expression of ZO-1 mRNA in LSCC and corresponding adjacent tissues. Compared with GAPDH internal control mRNA, the expression quantity of ZO-1 mRNA in LSCC tissues (0.0030±0.0014) was lower than in the adjacent nontumorous tis-
ZO-1 protein expression rate 51.1% (50/98) in tumor tissues and 100% (26/26) in adjacent noncancerous tissues was of statistical significance ($\chi^2 = 20.778$, $P = 0.000$). The rate of positive expression of ZO-1 in LSCC tissues is remarkably lower than that in the normal peritumoral tissue which was consistent with the qPCR results mentioned earlier.

**Relationship between ZO-1 protein expression and clinical parameters**

As for the correlation between ZO-1 expression and LSCC’s clinicopathologic factors (Table 1), it can be seen from the rank sum test and analysis that ZO-1 expression is related to the histopathological differentiation of carcinoma ($P = 0.009$), lymphatic metastasis ($P = 0.013$) and TNM classification ($P = 0.025$). In contrast, no significant correlation was found between sex, age, smoking, and tumor site.

**Correlation of ZO-1 protein expression and clinicopathologic parameters with lifespan**

Univariate analysis showed correlation of tumor location ($P = 0.003$), differentiation of tumor ($P = 0.000$), TNM stages ($P = 0.005$), lymphatic metastasis ($P = 0.001$) and ZO-1 expression level ($P = 0.001$) with lifespan of patients with LSCC. As indicated in the analysis of Cox proportional hazard regression model, the tumor location ($P = 0.030$), Histologic differentiation ($P = 0.004$), lymphatic metastasis ($P = 0.002$) and ZO-1 expression level ($P = 0.035$) were independent factors for bad prognosis of LSCC patients (Table 2).

In the high ZO-1 expression group among 98 LSCC patients, the total survival rate for 5 years is 82.0% (41/50), which is significantly higher
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than 58.3% (28/48) of the low ZO-1 expression group (P=0.015). Kaplan-Meier survival curves showed that the patients with low ZO-1 expression had a shorter survival time than those with high expression (Figure 3).

Discussion

LSCC is a common malignant tumor of head and neck. As the larynx is an important organ involving breath, pronunciation and deglutition, the growth and invasion of tumor will not only result in a patient’s loss of the above-mentioned functions but also severely threaten a patient’s life [15]. Therefore, searching for the biological markers of LSCC-related molecules for early diagnosis and accurate treatment is critically significant for raising the patient’s survival rate, reducing the full larynx removal rate and thus improving the patient’s post-treatment living quality.

The wholesome inter-epithelial connection is an important factor to maintain the epithelial stability. Researches show that ZO-1, which is the most important protein for adherent junction, has the adhesion and barrier functions [16]. In addition, ZO-1 also participates in the construction of cellular coupling function, can form a complex with ZO-2 and ZO-3, and forms the major closely-connected structural and functional protein through interconnections between its PDZ zone and other connexins.

Table 1. Relationship between ZO-1 expression and clinical parameters

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>n</th>
<th>Low expression</th>
<th>High expression</th>
<th>Z value*</th>
<th>P value*</th>
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<tr>
<td>Sex</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>96</td>
<td>48 (50.0)</td>
<td>48 (50.0)</td>
<td>-1.393</td>
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<td>Female</td>
<td>2</td>
<td>0 (0.00)</td>
<td>2 (100.0)</td>
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<td>Age, y</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;60</td>
<td>39</td>
<td>19 (48.7)</td>
<td>20 (51.3)</td>
<td>-0.042</td>
<td>0.967</td>
</tr>
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<td>≥60</td>
<td>59</td>
<td>29 (49.2)</td>
<td>30 (50.1)</td>
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</tr>
<tr>
<td>Smoking history (pack-years)</td>
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<td>&lt;60</td>
<td>31</td>
<td>14 (45.2)</td>
<td>17 (54.8)</td>
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<td>≥60</td>
<td>67</td>
<td>34 (50.7)</td>
<td>33 (49.3)</td>
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<td>Tumor site</td>
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<td></td>
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<td>Supraglottis</td>
<td>28</td>
<td>21 (75.0)</td>
<td>7 (25.0)</td>
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<td>0.361</td>
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<td>Glottis</td>
<td>70</td>
<td>27 (38.6)</td>
<td>43 (61.4)</td>
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<td>Histologic differentiation</td>
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<tr>
<td>Well</td>
<td>48</td>
<td>17 (35.4)</td>
<td>31 (64.6)</td>
<td>-2.618</td>
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<tr>
<td>Moderately and poorly</td>
<td>50</td>
<td>31 (62.0)</td>
<td>19 (38.0)</td>
<td></td>
<td></td>
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<td>TNM classification</td>
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<tr>
<td>I-II</td>
<td>62</td>
<td>25 (40.3)</td>
<td>37 (59.7)</td>
<td>-2.238</td>
<td>0.025</td>
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<td>III-IV</td>
<td>36</td>
<td>23 (63.9)</td>
<td>13 (36.1)</td>
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<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>81</td>
<td>37 (45.7)</td>
<td>44 (54.3)</td>
<td>-2.481</td>
<td>0.013</td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>11 (64.7)</td>
<td>6 (35.3)</td>
<td></td>
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</tr>
</tbody>
</table>

*The Z and p values were calculated by rank-sum test. Low and high expression were defined in immunohistochemical staining and scoring of materials and methods.

Table 2. Univariate and multivariate analysis of prognostic variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate*</th>
<th>Multivariate analysis†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>P value</td>
<td>HR 95% CI</td>
</tr>
<tr>
<td>Sex</td>
<td>0.744</td>
<td>2.206 0.496-9.804</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.456</td>
<td>0.825 0.491-1.388</td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td>0.506</td>
<td>1.095 0.576-2.081</td>
</tr>
<tr>
<td>Tumor site</td>
<td>0.003</td>
<td>2.046 0.732-5.715</td>
</tr>
<tr>
<td>Histologic differentiation</td>
<td>0.000</td>
<td>0.445 0.255-0.777</td>
</tr>
<tr>
<td>Stage</td>
<td>0.005</td>
<td>1.241 0.706-2.181</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.001</td>
<td>2.837 1.457-5.526</td>
</tr>
<tr>
<td>ZO-1</td>
<td>0.001</td>
<td>0.556 0.322-0.960</td>
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</tbody>
</table>

*Statistical analyses were performed by log-rank test. †Statistical analyses were performed by Cox regression model.
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Figure 3. Kaplan-Meier with laryngeal SCC by expression of ZO-1. Overall survival rate in patients with low expression of ZO-1 (blue line) was significantly lower than that in patients with high ZO-1 expression (green line).

such as actin [17-19]. Reichert M et al found that the β-catenin/Tcf/Lef signaling pathway was activated in the cells expressing the ZO-1 PDZ protein. The mutants of the ZO-1, which encode the PDZ domains (ZO-1 PDZ) but no longer localize at the plasma membrane, induced a dramatic epithelial to mesenchymal transition (EMT) of Madin-Darby canine kidney I (MDCKI) cells and lead to the occurrence of tumor [20].

We observed that both ZO-1 mRNA and protein levels were significantly lower in LSCC tissues than in the corresponding adjacent noncancerous tissues. Our study is the first to quantitatively analyze the expression of tight junction proteins in LSCC, and demonstrates that ZO-1 expression may play an important role in the LSCC tumorigenesis. Besides, higher degree of LSCC differentiation also indicates the higher expression of ZO-1 protein and the expression is low or absent in the poorly differentiated LSCC. This result is consistent with the research of small cell lung cancer [13] and, meanwhile, also proves that ZO-1 has the characteristics of epithelial marker, so it can be used as the opinion of indicators of the degree of epithelial differentiation [9]. In this test, the loss of ZO-1 expression is related to the decrease in differentiation degree and distant metastasis of tumor. On the one hand, ZO-1 is the major protein maintaining epithelial differentiation, so decrease in its expression will lead to the frustration of epithelial differentiation. On the other hand, ZO-1 can facilitate the cancer cells to reduce the epithelial polarity and obtain the polarity of interstitial cells. While the integrity of tight junction and adherent junction is lost, the construction of TJs is influenced to promote the occurrence of EMT in cancer cells [21]. The cell polarity malfunction will cause changes in epithelial morphology, reduce the intercellular adhesive force and strengthen its invasiveness. The permeability barrier malfunction will increase the permeability, help tumor cells to absorb abundant nutrient substances, thus promote the abnormal proliferation of tumor cells, and further increase the invasion as well as transfer ability of cancer cells [22].

In this research, the ZO-1 expression, tumor’s differentiation, clinical stages and lymphatic metastasis are independent factors affecting LSCC prognosis. As shown in Kaplan-Meier analyses, the 5-year survival rate of LSCC patients with high ZO-1 expression is notably higher than that of patients with low expression (P<0.05). This proved that ZO-1 has the functions to regulate the growth of tumor cells and facilitate the invasion and transfer of tumor cells [11]. Besides, the relation between the LSCC’s primary tumor location, lymphatic metastasis, clinicopathological stages and prognosis have been verified [23].

In this research, we made a relevant conclusion after making statistical analyses on the ZO-1 protein and LSCC clinical features by using the immunohistochemical method of tissue microarrays, so this conclusion has a certain limitation: firstly, there was a low quantity of samples especially the lymphatic metastasis samples; secondly, there was a lack of relevant cytological and animal in vivo experiments.

To sum up, we made a conclusion as follows: ZO-1 may be used as a prognostic factor in LSCC. ZO-1 may also represent a novel therapeutic target in LSCC. Further studies are required to investigate the biological functions of ZO-1 in LSCC.

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Disclosure of conflict of interest

None.

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References


