Original

Cytoplasmic ABCG2 and Podoplanin Expression in Oral Squamous Cell Carcinoma Correlates with Lymph Node Metastasis

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Abstract: The cervical lymph node metastasis of Oral Squamous Cell Carcinoma (OSCC) has been considered to be the main causes of death. The aim of our study was to clarify the expression pattern of ABCG2 and podoplanin in OSCC tissues and corresponding cervical lymph node, and evaluate the diagnostic value of ABCG2 and podoplanin in OSCC for tumorigenesis, histological stage and clinical prognosis. ABCG2 showed a mixed membranous and cytoplasmic pattern of staining in specific regions of stratum corneum and the center of well differentiated cancer nests, while only expressed in cytoplasm of poorly differentiated tumor nests. Podoplanin showed strong staining in LV endothelia, the periphery of well differentiated cancer nests, and tumor stromal fibroblasts surrounding tumor nests. The expression of ABCG2, especially cytoplasmic ABCG2, and podoplanin protein showed significantly higher tendency to lymph node metastasis (P<0.05). Meanwhile, podoplanin positive rate increased with the decreasing degree of histological differentiation (P<0.05). ABCG2 and podoplanin expression pattern is correlated with lymph node metastasis of OSCC. This result suggested that cytoplasmic ABCG2 with podoplanin have clinical potential in reliable molecule diagnosis for OSCC.

Key words: Cytoplasmic ABCG2, Oral squamous cell carcinoma, Lymph node metastasis, Podoplanin

Introduction

Head and neck squamous cell carcinoma (HNSCC) ranks the sixth in terms of incidence and mortality among all cancers throughout the world, with an estimated 300,000 new cases and 145,000 deaths in 20121. Oral squamous cell carcinoma (OSCC) is one of the most common malignant cancers of the head and neck regions, which has high cervical lymph node metastasis rate and recurrence rate. Owing to the occurrence and development of OSCC is a multi-stage and long-term complex process; the underlying molecular mechanisms of OSCC progression are still poorly understood.

ATP-binding cassette, subfamily G, member 2 (ABCG2), also known as ABCG2 and podoplanin (PDPN), is an important member of the superfamily of ATP-binding cassette transporter proteins. The ABCG2 transporter is widely distributed in the intestine, liver, kidney, and brain, and in addition mainly in the plasma membrane and cytoplasm. It is also known as the protein. Its functions include regulation of lymphatic vascular formation and platelet aggregation. A high level of podoplanin expression was correlated with an increased incidence of metastasis to lymph nodes and shorter survival time of patients.

The current study indicated that ABCG2 and podoplanin positive cancer cells in vitro shows highly correlated with the growth, metastasis and recurrence of cancer cells. Increasing evidence suggests that the expression of ABCG2 and podoplanin in oral lichen planus and oral erythroplakia were significantly associated with the risk of malignant transformation. However, there is a little evidence for the location and distribution of ABCG2 and podoplanin in OSCC tissues and corresponding cervical lymph node. To address these questions, we examined the expression pattern of ABCG2 and podoplanin of OSCC patients, and their implication for clinic-pathological features in OSCC.

Materials and Methods

Patients and clinical samples

Archival paraffin-embedded SCC tissue and corresponding cervical lymph node blocks from surgically resected oral squamous cell carcinomas and their corresponding clinical information (Table 1) were obtained randomly from 68 patients at Dalian Medical University Hospital in China and Okayama University Hospital in Japan between 2004 to 2011 after obtaining patients’ informed consent and approval by the Research Ethics Board at Dalian Medical University Hospital and Okayama University Hospital. Histological diagnosis of OSCC was...
Clinico-pathological features of OSCC (n=68)

<table>
<thead>
<tr>
<th>Age</th>
<th>Median (Y)</th>
<th>Mean (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>45 (66.18%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23 (33.82%)</td>
<td></td>
</tr>
<tr>
<td>Location (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>43 (63.23%)</td>
<td></td>
</tr>
<tr>
<td>Gingival</td>
<td>11 (16.18%)</td>
<td></td>
</tr>
<tr>
<td>Mouth floor</td>
<td>10 (14.17%)</td>
<td></td>
</tr>
<tr>
<td>Check</td>
<td>4 (5.88%)</td>
<td></td>
</tr>
<tr>
<td>Differentiation (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>36 (52.94%)</td>
<td></td>
</tr>
<tr>
<td>Moderate-poor</td>
<td>18 / 14 (47.06%)</td>
<td></td>
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<tr>
<td>LN metastasis (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN (-)</td>
<td>39 (57.35%)</td>
<td></td>
</tr>
<tr>
<td>LN (+)</td>
<td>29 (42.65%)</td>
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</table>

made according to the criteria of the World Health Organization for the histological typing of cancer and precancer of the oral. Diagnosis of the OSCC in this study was evaluated by head and neck cancer pathologists and informed consent was obtained from each patient.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded slides from either human tissue were used for the following IHC and quantitative analysis according the protocol we have described[^1]. The primary antibodies for human tissues used were ABCG2 (BXP-21) (1:50; Mouse monoclonal; abcam, Cambridge, UK), podoplanin (1:50; Mouse monoclonal; DAKO, Carpinteria, CA) and Ki-67 (1:50; Mouse monoclonal; DAKO, Carpinteria, CA). Identification of immunoreactive sites was achieved by subsequent incubation with a biotinylated secondary antibody, ABCG2 followed by the avidin-biotin complex (ABC) method (Vectorstain ABC Kit, Vector Laboratories, Burlingame, CA); podoplanin and Ki-67 followed by application of a commercial secondary antibody kit ( Envision HRP system, DAKO, Carpinteria, CA). For the negative control, sections were incubated by PBS instead of the primary antibodies.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics version 19.0 for Windows. Chi-square test and Fisher’s exact test were used to determine the positive percentage of tumor cells and clinicopathological characteristics. Correlation between ABCG2 and podoplanin expression in HNSCC were assessed by Spearman’s rank correlation analysis. All P-value of less than 0.05 were considered statistically significant.

Results

Clinicopathologic characteristics

45 male and 23 female OSCC patients with a median age of 64 (range 40-87) were included in this study. The tongue was most frequently affected (43/68, 63.23%), followed by the gingival (11/68, 16.18%), mouth floor (10/68, 14.71%) and cheek (4/68, 5.88%). The proportion of tumors with lymph node involvement was 42.65% (29/68). The clinic-pathological features of OSCC are summarized in Table 1.

Expression pattern of ABCG2 and podoplanin in OSCC

We investigated the localization and expression pattern of ABCG2 and podoplanin in OSCC. In order to examine the state of tumor cells, which shows podoplanin and ABCG2 protein expression, we choose Ki-67 to confirm cell proliferation.

Immunohistochemistry showed that the tumor cells in 35 (51.47%) of the 68 specimens were positive for ABCG2, the positive rate of cytoplasmic ABCG2 was 38.24% (26/68). The tumors cells in 52 (76.47%) of the 68 specimens were positive for podoplanin. ABCG2 and podoplanin staining were present mainly in the membrane and/or cytoplasm.

In the adjacent mucosa

Ki-67 expression was observed in the stratum germinativum (Fig. 1D). In most cases, all layers of epithelia did not express in ABCG2 (Fig. 1A), whereas in the specific regions of stratum corneum, the strong-positive protein reaction of ABCG2 can be observed (Fig. 1B). Podoplanin-positive cells were observed only in the stratum basale above lymphatic vessels (Fig. 1C). Podoplanin expression was observed in accordance with Ki-67 positive area. However, podoplanin expression is limited to more peripheral zone.

In the cancer nest

In well differentiated cancer nest, Membranous and cytoplasmatic ABCG2 expression was observed only in the center of cancer nest. In the basal-like layer and spinous-like layer of invading tumor nests, ABCG2 was low positive expression or no expression (Fig. 2A). Podoplanin was seen only around the basal-like layer of the invading cancer nest (Fig. 2B), showed the same pattern with the expression in Ki-67 (Fig. 2C). The tumor cells showed a strong podoplanin protein staining in its invasive front.

In poorly differentiated cancer nest, we can see Ki-67 was scattered in tumor cells (Fig. 2F). Meanwhile, cytoplasmatic ABCG2 (Fig. 2D) and podoplanin (Fig. 2E)-positive tumor cells were randomly.

In the lymph node

In 29 out of 68 cases of the patients involved with lymph nodes, we focus examined the expression of ABCG2 and podoplanin in these cases. In cancer nest within lymph node, Ki-67 showed high expression, it means the tumors cells were in high proliferative status (Fig. 2I). It is noteworthy that almost all cases of lymph node show ABCG2 (Fig. 2G) and podoplanin (Fig. 2H) strong membranous and cytoplasmatic staining, which showed the same expression pattern in LN (+) well differentiated cancer nest. (Fig. 2 G-I)
ABC2G2 (A) did not express in epithelia, however in the specific regions of stratum corneum, the strong-positive protein reaction of ABCG2 can be observed (B). Podoplanin (C) positive cells were observed only in the stratum basale above lymphatic vessels. Ki-67 (D) expression was observed in the stratum germinativum. The scale bars represent 100μm.

ABC2G2 (A) expression was observed only in the center of cancer nest. In poorly differentiated cancer nest, Ki-67 (F) was scattered in tumor cells. Meanwhile, ABCG2 (D) and podoplanin (E) were randomly positive. In well differentiated cancer nest within lymph node, the expression pattern of ABCG2 (G), podoplanin (H) and Ki-67 (I), are similar with LN(+) cancer nest in OSCC. The scale bars represent 100μm.
In well differentiated cancer nest, a strong membranous ABCG2 staining were observed in LN(-) cases (A), while a strong membranous and cytoplasmic ABCG2 staining of well differentiated cancer nest could be found in lymph nodes metastasis LN(+) cases (C); in poorly differentiated cancer nest, ABCG2 showed strongly cytoplasmic expression in LN(+) cases (D) than in LN(-) cases (B). The scale bars represent 50μm.

Table 3 Correlation of ABCG2 and podoplanin expression in OSCC

<table>
<thead>
<tr>
<th>ABCG2</th>
<th>Podoplanin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>+</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>52</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Cytoplasmic ABCG2</th>
<th>Podoplanin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>+</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>52</td>
</tr>
</tbody>
</table>

rs 4.565
p 0.022

Figure 3. Expression of markers in the LN(-/+ cancer nest in OSCC.

Correlations of ABCG2 and podoplanin markers expression with the patients' clinical variables

Correlations of ABCG2 and podoplanin expression with clinicopathological features of the OSCC patients are shown in Table 2. The expression of ABCG2 was no significant difference in the expression with the decreasing degree of histological differentiation. While, podoplanin positive rate increased with the decreasing degree of histological differentiation(P<0.05). No association was found, either, between markers expression and clinical parameters, such as the gender, age and location of OSCC patients(P>0.05). Only in lymph node metastasis cases, the expression of ABCG2, especially cytoplasmic ABCG2, and podoplanin protein showed significantly higher than those without lymph node metastasis cases (P<0.05). Immunohistochemistry staining results also showed that the LN(-) well differentiated cancer nest exhibited a strong membranous ABCG2 staining (Fig. 3A), while a strong membranous and cytoplasmic ABCG2 staining of well differentiated cancer nest could be found in lymph nodes metastasis cases (Fig. 3C). In poorly differentiated cancer nest, ABCG2 showed strongly cytoplasmic expression in lymph nodes metastasis cases (Fig. 3D), compare with LN(-) cancer nest (Fig. 3B). The spearman Rank-correlation analysis showed that the expression of podoplanin protein were positively correlated with ABCG2, especially cytoplasmic ABCG2, protein (Table 3, P<0.05)

Discussion

OSCC is the most frequently occurring cancer in the head and neck region and cervical lymph node metastasis is the main cause of death. Epidemiological data revealed that the overall survival at 5-year was ranging from 45% to 60% while a disappointing 24% in cervical lymph node metastasis patients. To our knowledge, expression pattern of ABCG2 and podoplanin in diverse differentiation stages and LN (+) tumor nests in primary tumors and corresponding cervical lymph node metastases has not been evaluated so far. Therefore, we examined ABCG2 and podoplanin expression in 68 cases of OSCC tissues and corresponding cervical lymph node. Our results revealed that the expression of ABCG2, especially cytoplasmic ABCG2, and podoplanin showed significantly higher expression in LN (+) primary tumors and corresponding lymph node metastases than those without lymph node metastasis cases (P<0.05). The expression of ABCG2, especially cytoplasmic ABCG2, was positively correlated to the expression of podoplanin.

Increasing evidence suggested that ABCG2 is recognized as a universal stemness marker and associated with tumor development and progression. To date, few studies have described the expression pattern and potential role of ABCG2 in OSCC. In our study, the results clearly reveal that ABCG2 showed two kinds of expression pattern. In some regions of stratum corneum and the central of invading well differentiated cancer nest, ABCG2 showed a mixed membranous and cytoplasmic pattern of staining, but in poorly differentiated tumor nests, ABCG2 only expressed in cytoplasm. Meanwhile, we also had discovered some interesting findings, comparing with LN(-
progression of head and neck cancer.

Current data has revealed that ABCG2 is involved in cancer lymph node metastasis. Tsunoda’s team immunohistochemically investigated paraffin sections of 100 esophageal squamous cell carcinoma (ESCC) tumors, ABCG2 level were associated with presence of lymph node metastasis. Patients with ESCC having ABCG2 expression may require more intensive or targeted therapy. It is generally known that ABCG2 is expressed mainly at the plasma membrane and cytoplasm in tumor cells. However, the significance of ABCG2 expression pattern in plasma membrane or cytoplasm is not yet clear. Wang XY et al found membranous ABCG2 expression in colorectal cancer correlated to lymph node and distant metastasis. While the cytoplasmic ABCG2 expression was only correlated with Dukes’ stage\(^{17}\), the standpoint which cytoplasmic ABCG2 is not bearing on the lymph node metastasis between us is discordant.

In our study, podoplanin, as a specific marker for lymphatic vessels, was expressed mainly in the cytoplasm and membrane of the tumor cells, it showed strong staining in LV endothelia, the periphery of well differentiated cancer nests, and tumor stromal fibroblasts surrounding tumor nests, especially in metastatic lesions in 68 OSCC tissues, this expression pattern is in accord with podoplanin+ cells in lung squamous cell carcinoma, head and neck, skin, uterine cervix cancer\(^{18-20}\). Expression pattern of ABCG2 with podoplanin illustrated that ABCG2 can be used as a sign to diagnosis of LN (+) OSCC.

As important potential biomarkers, there are no studies about ABCG2 and podoplanin co-expression in OSCC, only a few studies focus on oral precancerous lesions. Researchers have found that both ABCG2 and podoplanin positivity in oral erythroplakia and oral lichen planus were associated with the risk of oral cancer development and malignant transformation\(^{21-23}\). Our data showed that in poorly differentiated cancer nest and LN (+) well differentiated cancer nest, podoplanin shows strong expression and the overlap among some podoplanin+ and ABCG2+, especially cytoplasmic ABCG2+, which indicated that ABCG2, especially cytoplasmic ABCG2, and podoplanin may be important factors related to tumor differentiation and cervical lymph node metastasis of OSCC.

In summary, our immunohistochemical results demonstrates that ABCG2, especially cytoplasmic ABCG2, and podoplanin are commonly expressed in poorly differentiated cancer nest and LN (+) well differentiated cancer nest. According to current study, cytoplasmic ABCG2 and podoplanin strong staining have clinical potential in reliable molecule diagnosis for oral squamous cell carcinoma. In addition, it would be worthwhile to search for the molecular mechanism of ABCG2 with other canonical oncogenes related to lymph node metastasis of OSCC.

Acknowledgements

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Conflict of interest statement

None of authors have conflict of interest to declare.

References


