Expression of Claudin-1 in Laryngeal Squamous Cell Carcinomas (LSCCs) and Its Significance

Authors: Abderrahman Ouban

DOI: 10.14670/HH-18-320
Article type: ORIGINAL ARTICLE
Accepted: 2021-02-25
Epub ahead of print: 2021-02-25

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed. Pre-print author’s version
Expression of Claudin-1 in Laryngeal Squamous Cell Carcinomas (LSCCs) and Its Significance.
Abstract

Background:
A large body of scientific evidence points to the important roles of tight junction proteins in tumor development, progression and dissemination. The larynx has only a few studies, analyzing the role of this group of junctional proteins in its oncogenesis. In this study, the author sheds some light on the expression and possible role of claudin-1 in laryngeal squamous cell carcinomas.

Materials and Methods:
This study analyzed the expression of claudin-1, using immunohistochemistry, in a tissue microarray of 80 cases of laryngeal squamous cell cancers. Clinicopathological parameters were analyzed according to claudin-1 expression in the tissue microarray. Furthermore, the expression of slug/snail1, an Epithelial-Mesenchymal Transition (EMT) linked protein, was analyzed by immunohistochemistry in the same microarray, and the expressions of the two proteins were assessed for correlation.

Results:
A significant majority of laryngeal squamous cell cancers exhibited positive expression of claudin-1 proteins. The majority of those tumors expressed claudin-1 in their cytoplasm. The overall majority of those same tumors also exhibited a cytoplasmic shift of the slug-snail-1 protein from the nuclei to the cytoplasm. There was also evidence of correlation of the two proteins’ expressions in the cytoplasm of laryngeal tumors.

Conclusion:
The above may suggest a role for claudin-1 in the development and progression of laryngeal squamous cell carcinoma. Overall, claudin-1’s aberrant expression in laryngeal cancer is in line with evidence seen in other head and neck cancers. Its co-expression with slug/snail-1 in LSCC patients should be investigated further to understand the nature of the relationship of the two proteins in LSCC and their possible contribution to its development and progression.
Introduction:

Tight junction strands are the structures which connect cells side-by-side, regulating the paracellular transport, maintaining cell polarity and protecting the underlying cellular structures. By playing the roles of a guard and a seal, tight junctions (TJs) exert selective permeability of solutes through the cellular membranes (Anderson et al., 2009). These structures are dynamic entities which, through their weak and short-lived interactions with each other, provide a model where breaking and resealing the TJ strands regulate the paracellular diffusion of solutes (Lim et al., 2008). The backbone of these strands is a group of 27+ transmembrane proteins, known as the claudin proteins (Morita et al., 1999; Morin, 2005; Sawada, 2013).

While these roles are critical for cell survival and homeostasis, the claudins’ role in carcinogenesis has more to do with their roles in signal transduction (Swisshelm et al., 2005), in recruiting and enhancing the activation of pro-matrix metalloproteinase 2 (Miyamori et al., 2001; Schmalhofer et al., 2009), and in being regulated by nuclear transcription factors seen with claudin-1 (Miwa et al., 2000), where altered cell adhesion will modulate gene activity via cellular junctional-to-nuclear pathway such as the β-catenin signalling (McCrea et al., 2009).

In head and neck cancer, claudin-1 is featured prominently, where the majority of studies from that region have shown that claudin-1 over-expression is associated with tumor development, progression and metastases (Dhawan et al., 2005; Dos Reis et al., 2008; Miyamoto et al., 2008; Ouban and Ahmed, 2015; Li et al., 2015; Zwanziger et al., 2015; Gucer et al., 2016; Babkair et al., 2016; Wu et al., 2018; Aoyama et al., 2019; Qiu et al., 2019). However, a few studies have addressed the expression of claudin-1 in tumors from the larynx region. One such recent study (Zhou et al., 2019) has pointed out a decrease in the level of claudin-1 in laryngeal squamous cell cancers. While it is logical to expect that levels of claudins will decrease with carcinogenesis and metastases, the majority of sites in the head and neck region have reported increased expressions of claudin-1, which when linked to clinical-pathologic parameters were shown to be associated with worse prognosis of cancer (Dhawan et al. 2005; Dos Reis et al., 2008; Miyamoto et al., 2008; Ouban and Ahmed, 2015; Li et al., 2015; Zwanziger et al., 2015; Gucer et al., 2016; Babkair et al., 2016; Wu et al., 2018; Aoyama et al., 2019; Qiu et al., 2019).

Furthermore, one recent study (Li et al., 2015) has shown that claudin-1 levels were significantly higher in hypopharyngeal SCC, and those levels were related to tumor differentiation and lymph node metastases. This is an interesting finding, given that the two sites, the hypopharynx and the larynx, share embryological, anatomical and histological common origins. In their work, Zhou and co-workers, did not report the method of scoring of the claudin-1 protein in their samples, nor did they provide any reference of the same. (Zhou et al., 2019) This current study was performed to provide accurate assessment of claudin-1 levels in laryngeal cancer cells, and to assess concurrent expression of claudin-1 and slug/snail-1, both linked to the EMT phenomenon and tumor cells’ aggressiveness.
Materials and Methods.

Tissues.

Two (2) commercial human larynx microarrays (Catalogue no. LP803; Biomax US, Rockville, MD), with 80 cases of laryngeal squamous cell carcinomas each, were used for this study. All the cases were confirmed laryngeal squamous cell carcinomas. Major parameters of these tumors include age, sex, anatomic site, pathologic diagnosis and grade. For comparison with normal laryngeal mucosa, a second microarray (Catalogue no. FDA 992, Biomax US, Rockville, MD) carrying normal tissues from all human anatomic sites was used. A normal benign skin tissue, obtained from an unrelated patient, was used as a positive control for claudin-1 expression. The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Alfaisal University.

Immunohistochemical (IHC) Stains

For immunostaining, the slides were deparaffinized and epitopes were retrieved using Dako Retrieval Solution (Dako Cytomation, USA) at 95°C for 30 minutes, followed by cooling to room temperature for 2 minutes. 0.3% H2O2 was used to inactivate endogenous peroxidase. Subsequently, the sections were rinsed twice in PBS for 5 minutes. Immunostaining was done with antibodies directed against claudin-1 (rabbit, polyclonal, ab 15098; abcam, UK), and slug/snail-1 (rabbit, polyclonal, ab 180714, abcam, UK); following dilution in antibody diluent (Agilent, Santa Clara, California). The Vectastatin ABC peroxidase kit was used according to the manufacturer’s instructions (Vectastatin Elite ABC Kit, Vector Laboratories, USA). Negative controls were used with omission of primary antibody. Separate positive controls of normal skin were used to test optimization and run validation (Ouban and Ahmed, 2015).

Staining Evaluation

The IHC slides were evaluated by the PI (AO). The degree of claudin-1 reactivity was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system as described by Baccelli and coworkers (Baccelli et al., 2014). The staining intensity (SI) was categorized into four grades: 0, no immunostaining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of positive cells (PP) was categorized into five grades: 0 (0%); 1, (1–10%); 2 (11–50%); 3 (51–80%); and 4 (>80%). The staining intensity and percentage of positive cells were multiplied to obtain an IRS, in the range of 0–12 for each individual case. A case was scored as positive for claudin-1 with an IRS between 7 and 12, and negative with an IRS between 0–6 (Baccelli et al., 2014). The degree of slug/snail-1 reactivity was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system, as described by Remmele and co-workers (Remmele et al., 1986). The staining intensity (SI) was categorized into 4 grades: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positive cells (PP) was regarded as 0 (none), 1 (≤10%), 2 (11–50%), 3 (51–80%) and 4 (>80%) positive tumor cells. The product of SI and PP is the IRS (0–12). A score of 0–2 was regarded as negative, while a score of 3–12 was regarded as positive (Remmele et al., 1986).
**Statistical Analysis:**

The $\chi^2$ test was used to compare the claudin-1 expression between laryngeal cancer tissue and benign laryngeal tissue, analyze the association between claudin-1 expression and the clinicopathologic parameters including gender, age, and differentiation grade and evaluate the correlation between claudin-1 expression and slug/snail-1 EMT marker. All statistical analyses were performed with IBM SPSS Statistics software package, version 25.0. $p<0.05$ was considered to indicate a statistically significant difference.

**Results**

The human larynx microarray (Catalogue no. LP803; Biomax US, Rockville, MD) with 80 cases of laryngeal squamous cell carcinomas was used for this study. The age of the cancer patients in this array was in the range of 39–72 years, with a mean of 53 years. LSCC grades were distributed as follows: 25 grade I; 43 grade II and 7 were grade III. Five tumors in the microarray did not have a grade designation reported by the manufacturer of the array. The expression of claudin-1 in normal, benign, unmatched laryngeal mucosa (Catalogue no. FDA 992, Biomax US, Rockville, MD) was apical, membranous and faint or absent (IRS# 0-2, Fig.1d). In laryngeal SCC cases, on the other hand, 64/80 cases were positive for claudin-1 (80% with an IRS of 7-12, $\chi^2= 28.800; p<0.001$, Fig. 2 A-E, Table 1). The rest of the cases (16 in total) had weak or negative claudin-1 expression (IRS# 0-2). A significant majority ($p<0.001$) of laryngeal SCC cases (87.5% of cases, $p<0.001$) expressed claudin-1 in a combined pattern in the cytoplasm and nuclei of the tumor cells (Fig. 3A-D, Table 1).

**Association between claudin-1 expression and clinic-pathological parameters of LSCCs.**

Positive expression for claudin-1 was significantly correlated with LSCC grade-differentiation (Table 2, $\chi^2= 20.885$, df=2; $p<0.001$). There was no significant association between positive expression for claudin-1 and gender or age (both were at $p>0.05$).

**Association between claudin-1 and slug/snail-1**

Among the LSCC cases, 71 were positive for slug/snail-1 immunostain. Sixty-three of 64 (63/64) claudin-1 positive cases were also positive for slug/snail-1, among them 54 cases were positive in the nuclei and cytoplasm and 9 had nuclear expression of slug/snail-1 ($\chi^2= 74.246$; df=2; $p<0.001$). Only one case was positive for claudin-1 and negative for slug/snail-1. Seven cases were positive slug/snail-1 and negative for claudin-1 (Fig. 4 A-B represents expression of slug/snail-1 in normal unmatched laryngeal mucosa tissue: Nuclear expression is seen. Fig 5. A-D: Represents combined nuclear/cytoplasmic expression of slug/snail-1 in LSCC cells).

**Discussion**

Claudin-1 expression has been recently implicated as one of the important events of carcinogenesis in several human cancers (Miwa et al., 2000; Dhawan et al., 2005; Dos Reis et al., 2008; Schmalhofer et al., 2009; Bezdekova et al., 2012; Kinugasa et al., 2012; Stebbing et
al., 2013; Cunniffe et al., 2014; Huang et al., 2014; De Vicente et al., 2015; Li et al., 2015; Ouban and Ahmed, 2015; Wan et al., 2015; Huang et al., 2015; Zwanziger et al., 2015; Zhou et al., 2015; Guce et al., 2016; Majer et al., 2016; Babkair et al., 2016; Tas et al., 2016; Hahn-Stromberg et al., 2017; Ouban, 2018; Wu et al., 2018; Qiu et al., 2019; Aoyama et al., 2019). In the present study we have documented the positive expression of this protein in a significant majority of LSCC cases. Claudin-1 expression also showed significant differential expression based on the grade of the LSCCs. Claudin-1 was statistically more likely to be expressed in a combined nuclear/cytoplasmic position in LSCCs cells than the normal membranous position. This subcellular displacement of claudin-1, away from where it is naturally expressed on the basolateral aspect of the cell membrane (Morita et al., 1999) to the cytoplasm, and sometimes to the nucleus, has been seen with other tumors, including colorectal, oral, ovarian and breast cancers (Dhawan et al., 2005; Zhu et al., 2006; Dos Reis et al., 2008; Bezdekova et al., 2012; De Aquino et al., 2012; Ouban and Ahmed, 2015; and Ouban, 2018). While the role of this claudin-1 cytoplasmic shift was first thought to be related to cell-matrix interaction and vesicle trafficking (Blackman et al., 2005), more evidence showed it to result in increased paracellular permeability (Morin et al., 2005).

The role of claudin-1 expression in head and neck cancer is well documented, and involves in-vitro as well as in-vivo evidence. For example, it was found that the level of expression of claudin-1 correlated well with the activity levels of both MMP2 and MMP9; and was found to enhance cleavage of laminin-5 gamma2 chains in oral squamous cell cancer, thus facilitating invasion (Oku N et al., 2006; Lal-Nag et al., 2009). Claudin-1 expression was associated with downregulation of E-cadherin, upregulation of vimentin and attainment of aggressive phenotype in oral SCCs (Oku et al., 2006). Furthermore, Wu and co-workers found that claudin-1 gene positive expression promotes the proliferation, invasion and migration of nasopharyngeal cell line cells through up-regulation and nuclear entry of β-catenin (Wu et al., 2018). Claudin-1 expression was high in hypopharyngeal squamous cell carcinoma, and significantly correlated with tumour differentiation, micro-lymphatic vessel density (MLVD) in cancer tissues, and lymph node metastases (Li et al., 2015). The same study showed that claudin-1 over-expression was significantly related to patient survival rate (Li et al., 2015). Claudin-1 over-expression was found to significantly influence the behaviour of follicular thyroid cancer cells, where cell lines of metastases of follicular thyroid carcinomas were found to have a significantly higher claudin-1 protein expression in the nucleus compared to other non-metastatic cell lines (Zwanziger et al., 2015). The authors showed that transfecting follicular thyroid cancer cells with a vector carrying the claudin-1 gene resulted in enhanced cell migration, proliferation and invasion (Zwanziger et al., 2015). Metastatic lower lip squamous cell carcinomas (LLSCCs), another tumor in the head and neck region, exhibited significantly higher expression of claudin-1 than non-metastatic tumours (De Aquino et al., 2012). Furthermore, the authors found that advanced stages (III and IV) of the LLSCCs showed significantly higher levels of claudin-1 than stages I and II. The above presents evidence of claudin-1 involvement in aggression, invasion, metastases and poor survivability in head and neck cancers. This current study completes the above evidence by documenting the positive expression of claudin-1 and its clinicopathologic significance in LSCCs.

Interestingly, a study of colorectal cancer (Dhawan et al., 2005) showed that claudin-1 membrane-to-cytoplasm switch was associated with induction of the epithelial-mesenchymal transition (EMT) phenomenon in cancer cell lines. To further investigate the role of claudin-1 in the EMT phenomenon in LSCC, the expression of a protein known for its role in the EMT phenomenon, slug/snail-1, was analyzed in the same patients’ sample. While slug/snail-1 was expressed in both normal and malignant tissues, this study presents significant differences in the subcellular localization of slug/snail-1 between normal and malignant laryngeal tissues.
The protein expression was nuclear in normal unmatched laryngeal tissues (Fig.4); however, LSCC cells significantly expressed the slug/snail-1 protein in a combined nuclear/cytoplasmic pattern (Fig.5). This aberrant slug/snail-1 expression was significantly correlated with claudin-1 expression (Table 3, p<0.001). Slug/snail-1 is a transcriptional protein, which plays important roles in cellular proliferation, differentiation and apoptosis (Bezdekova et al., 2012). In the pathologic state, it plays a multirule as an oncogene in intestinal tumorigenesis (Roy et al., 2004), a transcriptional repressor of E-cadherin and a potent inducer of the epithelial-mesenchymal transition phenomenon (EMT) in many types of human cancers (Chang et al., 2012; Lee et al., 2014; Lin et al., 2014 and Liu et al., 2015). Snail-1 acts in-tandem with other EMT transcription factors (EMT-TFs), such as “twist”, to transcriptionally repress the E-boxes present in the E-cadherin promoter resulting in its down-regulation (Serrano-Gomez et al., 2016).

Besides the co-expression seen between the two proteins in this study’s tumors, there are other facets to the relationship between them. Martinez-Estrada and co-workers, using immunofluorescence microscopy, found diminished levels of claudin-1 in MDCK-slug/snail-1 transfected cells, with redistribution of the protein from the TJ to the cytoplasm of those cells (Martinez-Estrada et al., 2006). These authors and others (Ikenouchi et al., 2003), using transfection experiments and electrophoretic mobility assays, reported that this reduction in claudin-1 levels is traced back to two E-box motifs found in the human claudin-1 promoter; which conform to two snail-binding E-boxes (Ikenouchi et al., 2003; Martinez-Estrada et al., 2006). They were able to confirm that snail-1 binds directly to the E-boxes of the promoters of the claudin-1 gene, resulting in complete downregulation of its activity. With this discovery, it was confirmed that the transcription promoter activity of human claudin-1 is directly regulated by slug/snail-1 (Ikenouchi et al., 2003; Martinez-Estrada et al., 2006).

By contrast, claudin-1 overexpression resulted in upregulation of slug and induction of EMT in liver cancer Chang cell lines (Suh et al., 2013). The authors found that claudin-1 induces EMT through Ras-Raf-1-ERK signaling in Chang cells. Treatment of claudin-1-expressing Chang cells with si-RNA directed against claudin-1, resulted in the resolution of the EMT phenomenon and normalization of its markers levels (Suh et al., 2013). So while the expectation is for claudin-1, along with other tight junction proteins, expression to decrease during the EMT process (Ikenouchi et al., 2003; Ohkubo et al., 2004), recent studies have shown that EMT could happen with a high expression of claudin-1 (Bhat et al., 2016; Lv et al., 2017). The latter finding may support a signaling role for claudin-1 in the EMT phenomenon (Suh et al., 2013), or may result from a mutated claudin-1 gene, resistant to downregulation by the E-box motifs of snail-1 (Martinez-Estrada et al., 2006).

The above should provide a platform for future research directions in order to analyze the role of claudin-1 in LSCC, to investigate claudin-1/slug-snail-1 interactions in LSCC and to provide answers regarding the contribution of this protein in LSCC development and behavior.

**Conclusion:**

In line with other tumors in the head and neck in general, this study presents evidence of a significant expression of claudin-1 in LSCC tumor cells and shows that this expression is correlated with both tumors grade, and slug/snail-1 expression. Future research is in order, to investigate the role of claudin-1 in LSCC and to analyze the nature of the interaction between claudin-1 and slug/snail-1 in this tumor and to provide a detailed map of how the two proteins may collaborate in laryngeal tumorigenesis and progression.
• No funding was provided for this study
• The author reports no conflict of interest.

References


by promoting cleavage of laminin-5 gamma2 chain via matrix metalloproteinase (MMP)-2 and membrane-type MMP-1. Cancer Res. 66, 5251-5257.


### Tables.

**Table 1.** Claudin-1 expression in laryngeal squamous cell cancers and its subcellular location.

<table>
<thead>
<tr>
<th>Claudin-1 expression and position in cancer cell</th>
<th>All cases, N</th>
<th>%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Claudin-1 Expression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEG</td>
<td>16</td>
<td>20</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>POS</td>
<td>64</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td><strong>Subcellular localization of claudin-1 in positive tumor cell</strong></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Pure membranous</td>
<td>8</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic/Nuclear</td>
<td>56</td>
<td>87.5</td>
<td></td>
</tr>
</tbody>
</table>

NEG, negative; POS, positive. *: indicates a statistically significant result.

**Table 2: Correlation analyses between the expression of claudin-1 and clinicopathologic features.**

<table>
<thead>
<tr>
<th>Correlation analyses</th>
<th>All cases, N</th>
<th>%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>81</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>&gt;53</td>
<td>42</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>&lt;53</td>
<td>22</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Differentiation Grade</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>W. diff. (G1)</td>
<td>22</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>M. diff. (G2)</td>
<td>34</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>P. diff. (G3)</td>
<td>5</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

G1, grade 1; G2, grade 2; G3, grade 3. *: indicates a statistically significant result.

# It is noted that the number of graded tumors is 61. Three (3) cases were positive for claudin-1, but were not assigned grades in the commercially provided tissue microarray (TMA) by the manufacturer.

**Table 3.** Correlation analyses between claudin-1 positive cases, and slug/snail-1 positive expression

<table>
<thead>
<tr>
<th>Correlation between CLDN and Slug/Snail-1</th>
<th>All cases, N</th>
<th>%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail/Slug Positive Expression</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Nuclear only</td>
<td>9</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Nuclear + Cytoplasmic</td>
<td>54</td>
<td>84</td>
<td></td>
</tr>
</tbody>
</table>

*: indicates a statistically significant result