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ORIGINAL PAPER

Overexpression of Claudin-1 is Associated with Advanced Clinical Stage and Invasive Pathologic Characteristics of Oral Squamous Cell Carcinoma

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Abstract Claudins constitute a group of principal proteins forming the tight junctional complex. The altered expression of selected claudins has been reported in several human cancers. The purpose of this study was to investigate the expression of claudin-1 and claudin-4 in oral squamous cell carcinoma (OSCC) and examine its relationship with patient clinical-pathologic features. Forty-five OSCC cases were enrolled. Patient clinical, pathologic and follow-up data were reviewed and the claudin-1 and claudin-4 expression was analyzed immunohistochemically. Positive claudin-1 and claudin-4 immunoreactivities were noted in 86.7 and 80 % of cases, respectively. The majority of cases showed the staining in less than 25 % of cancer cells. The increased claudin-1 expression was significantly associated with the high pathologic grade, the presence of microscopic perineural invasion, vascular invasion, nodal metastasis, and advanced clinical stage. No relationship between various clinico-pathologic parameters and differential claudin-4 expression was observed. Claudin-1 may play a role in OSCC progression and could serve as a prognostic marker of advanced disease.

Keywords Claudin-1 · Claudin-4 · Oral squamous cell carcinoma · Prognosis

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Introduction

Oral cancer is one of the most prevalent cancers worldwide and accounts for the eighth most common cause of cancer-related deaths. The squamous cell carcinoma (SCC) comprises over 90 % of all oral cancers [1]. The molecular mechanisms of oral squamous cell carcinoma (OSCC) have been widely studied in an attempt to better understand the underlying pathogenesis of this malignancy and potentially improve the prognosis and treatment outcome for patients. Despite ongoing research to uncover novel prognostic indicators and therapeutic agents, the mortality rate of patients with this cancer has remained unchanged [2].

One distinctive feature of all epithelial tissues and epithelial neoplasms is that epithelial cells adhere together in cellular sheets through the junctional complex, formed by tight junctions, adherens junctions and desmosomes. Apart from this fundamental task, tight junctional proteins are also responsible for regulating paracellular ion permeability and maintaining cellular polarity [3]. In addition, recent studies also showed that these proteins could be involved in regulating the signalling mechanisms and controlling the cellular proliferation and differentiation [4].

The claudin family of proteins represents the principal protein assembly of the tight junctional complex. It consists of 24 closely related transmembrane proteins which are differentially expressed in various types of tissue [5, 6]. Most tissues also express more than one type of claudin.

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Claudin-1 and claudin-4 in particular have been shown to be critical for the function of tight junctions [7].

The altered expression of selected members of the claudin family has been reported in several cancers [8, 9]. Their levels of expression appear to be distinctly variable depending on the type of cancers. The claudin-1 expression was shown to be down-regulated in breast and prostate cancers [10–12], whereas its overexpression was observed in gastric, thyroid, pancreatic, urothelial and cervical cancers [13–16].

The claudin-4 expression was up-regulated in several cancers, including esophageal, gastric, colorectal, pancreatic, ovarian, urothelial, breast, endometrial and prostate cancers [12, 14, 17–23]. Its role in the invasiveness and survival of ovarian cancer cells has also been suggested in an in vitro study [24].

A handful of studies examined the changes in claudin expression in OSCC [25–31]. The potential role of claudin-1 in promoting the growth and invasion of OSCC cells was reported [32–34]. However, no study has shown the association of claudin-1 and claudin-4 protein expression in OSCC and clinical prognostic factors. Therefore, the objective of this study was to investigate the expression of these two members of the claudin family in OSCC and the relationship with the prognostically-related clinical-pathologic features.

Materials and Methods

Tissue Samples

OSCC archival cases from the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand between 2003 and 2013 were retrieved. The inclusion criteria of subjects were applied to include the OSCC patients with (1) primary tumor of the intraoral sites (tongue, buccal mucosa, gingiva, alveolar mucosa, floor of mouth and hard palate); (2) adequate medical records and follow-up data; and (3) sufficient paraffin-embedded tissue specimens. Patient data collected were the age, sex, anatomical site, tumor size, nodal or distant metastatic status, treatment received, recurrence and survival information. The clinical staging of lesions was based on the TNM classification of the American Joint Committee of Cancer. Patients with unresectable tumor or tumor at multiple sites were excluded from this study.

Overall, 45 cases of OSCC were enrolled. All microscopic slides were reviewed to confirm the diagnosis and the histopathologic grading was performed based on the World Health Organization criteria. The histopathologic parameters were assessed and agreed upon by two pathologists. The study was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University.

Immunohistochemical Methods

The immunohistochemical staining was performed solely on the primary lesions with Leica Microsystems Bond-Max Autostainer System. The 5 μm-thick sections were deparaffinized with the Bond Dewax Solution. The antigen retrieval was performed for the claudin-1 staining by incubating slides with the Bond Epitope Retrieval Solution 2 for 30 min at 95 °C. For the claudin-4 staining, slides were incubated with the Bond Epitope Retrieval Solution 1 for 20 min at 95 °C.

The primary antibodies used were polyclonal anti-claudin-1 (1:200 dilution) and monoclonal anti-claudin-4 (1:500 dilution) antibodies (Invitrogen, Camarillo, CA). The immunohistochemical procedure was performed using the Bond Polymer Refine Detection kit (Leica Microsystems), a 3-step indirect immunoperoxidase technique. Briefly, after 5-min application of 3 % hydrogen peroxide, sections were incubated with the primary antibodies for 50 min at room temperature. The Post Primary Polymer was then added for 12 min, followed by additional 12-min incubation with the Polymer Poly-HRP IgG. The sections were incubated with diaminobenzidine chromogen for 3 min and counterstained with haematoxylin. The Bond Wash Solution was used to rinse between each step. As positive controls, colonic mucosa samples were used. Negative controls were prepared using the isotype-matched antibodies. The rabbit IgG and mouse IgG1 were applied instead of the anti-claudin-1 and anti-claudin-4 antibodies, respectively.

Immunohistochemical Assessment and Statistical Analysis

The immunohistochemical assessment was performed and agreed upon by two pathologists, who were blinded to all patient clinical and follow-up data. The positive immunoreactivity localized on the plasma membrane of neoplastic cells was evaluated. Overall, the percentage of positive neoplastic cells was semi-quantitatively assessed and categorized into one of the following groups: 0 = no positive cells; 1+ = positive cells detected in less than 25 % of tumor; 2+ = positive cells detected between 26 and 50 % of tumor; 3+ = positive cells detected between 51 and 75 % of tumor and 4+ = positive cells detected in more than 75 % of tumor. The expression of claudin-1 and claudin-4 was further classified as low immunoreactivity (groups 0, 1 and 2) and high immunoreactivity (groups 3 and 4). The immunostainings of the entire cancer tissues as

Table 1 Clinico-pathologic features of 45 OSCC patients

Clinical-pathologic variables	Number of patients [n (%)]
Sex	
Male	22 (48.9)
Female	23 (51.1)
Age (years)	
Mean ± SD (65.82 ± 12.10)	
Range (44–86)	
Site	
Gingiva	17 (37.8)
Tongue	7 (15.6)
Floor of mouth	8 (17.8)
Buccal mucosa	6 (13.3)
Hard palate	3 (6.7)
Alveolar mucosa	4 (8.9)
T stage	
T1	24 (53.3)
T2	11 (24.4)
T3	5 (11.1)
T4	5 (11.1)
N stage	
N0	21 (46.7)
N1	13 (28.9)
N2	7 (15.6)
N3	4 (8.9)
Distant metastasis	
Absent	43 (95.6)
Present	2 (4.4)
Recurrence	
Absent	35 (77.8)
Present	10 (22.2)
Pathologic grade	
Well differentiated	24 (53.3)
Moderately differentiated	15 (33.3)
Poorly differentiated	6 (13.3)
Perineural invasion	
Absent	28 (62.2)
Present	17 (37.8)
Vascular invasion	
Absent	26 (57.8)
Present	19 (42.2)
TNM staging	
Stage I	18 (40.0)
Stage II	6 (13.3)
Stage III	7 (15.6)
Stage IV	14 (31.1)

well as the invasive fronts were evaluated. The invasive tumor front was determined by the tumor cells invading through the underlying muscular layer.

The results were statistically analyzed using the IBM SPSS Statistics version 21 (IBM Corporation, NY) for Windows. The continuous variables were expressed as mean ± standard deviation (SD). Categorical analyses of the clinico-pathologic parameters and the claudin-1 or claudin-4 expression were performed using either the Pearson's Chi square test or the Fisher's exact test, as appropriate. A P value less than 0.05 was considered statistically significant.

For statistical analysis, the clinico-pathologic features were grouped as following: age below or above 65 years (the mean age of patients); T stage IV or below; early TNM stage (I, II) or late TNM stages (III, IV); microscopically well differentiated or moderately/poorly differentiated; the presence of perineural invasion, vascular invasion, local recurrence, regional lymph node involvement and distant metastasis.

The disease-specific survival was determined as the time following surgical operation to the time when the patient died of cancer. Univariate analysis was performed using the Kaplan–Meier method and the log rank test was used to analyze the significant differences between groups.

Results

Patient Characteristics

The clinical and pathological characteristics of 45 OSCC patients are presented in Table 1. Briefly, there were 22 male and 23 female patients with a mean age of 65.82 + 12.10 years (range 44–86 years). The majority of lesions were located on gingiva (37.8 %), followed by floor of mouth (17.8 %), tongue (15.6 %) and buccal mucosa (13.3 %). Ten patients reported local recurrence (22.2 %). Twenty-four patients (53.3 %) had regional lymph node involvement, and 2 patients had distant metastasis (4.4 %). The majority of patients were classified as TNM stage I (40.0 %), followed by stage IV (31.1 %). Microscopically, 53.3 % of cases were graded as well-differentiated, followed by moderately differentiated (33.3 %) and poorly differentiated (13.3 %).

Expression of Claudin-1 and Claudin-4 in OSCC

A membranous staining pattern was noted in all positive cases of both proteins. In most cases, the central squamous cells of tumor nests showed more intense staining than the peripheral basal cells. However, some cases also demonstrated strongly positive staining at the periphery of tumor islands (Fig. 1). In general, the immunostaining of the invasive tumor fronts was similar to that of the bulk of specimens, with the exception of only 3 cases which showed the slightly increased percentage of claudin-1 positive

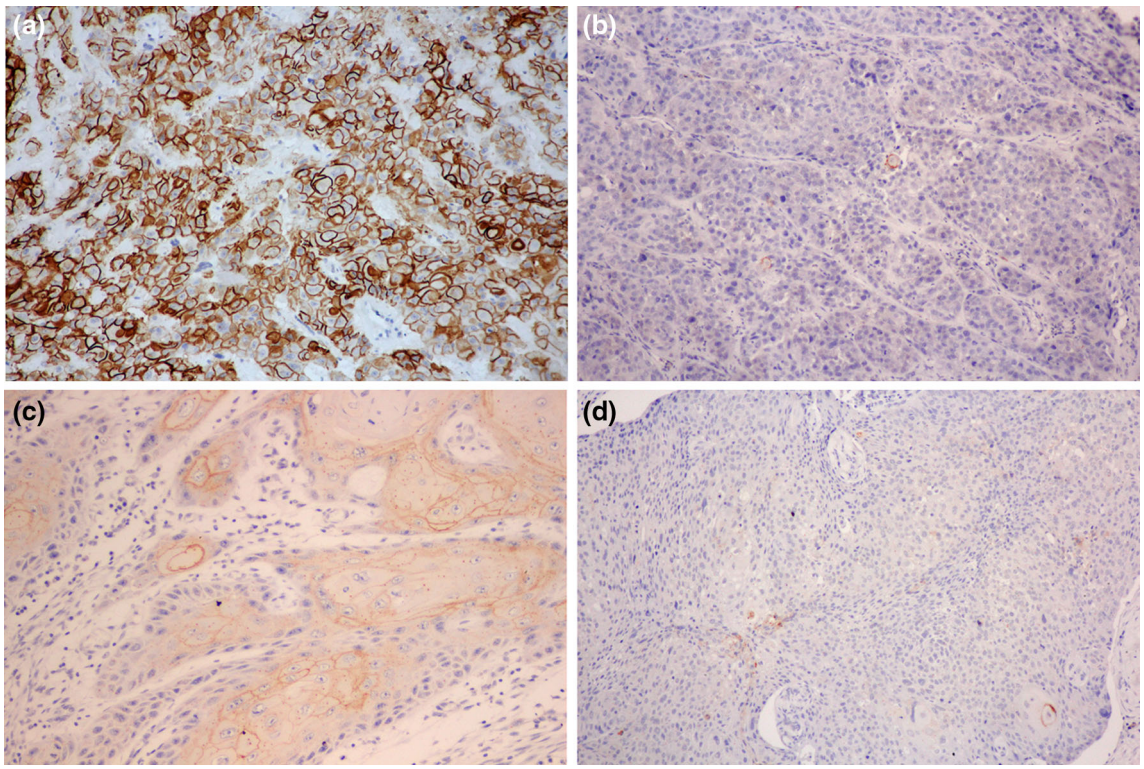

Fig. 1 Representative photomicrographs of claudin-1 and claudin-4 immunoreactivity in OSCC (Magnification ×100). **a** High claudin-1 expression; **b** Low claudin-1 expression; **c** High claudin-4 expression; **d** Low claudin-4 expression

Table 2 Differential expression of claudin-1 and claudin-4 in OSCC

	Immunohistochemical staining in neoplastic cells [n (%)]					Spearman's rho	
	0	1+	2+	3+	4+	r	*P* value
Claudin-1	6 (13.3)	15 (33.3 %)	12 (26.6 %)	3 (6.7 %)	9 (20.0 %)	0.32	0.03
Claudin-4	9 (20.0 %)	30 (66.7 %)	2 (4.4 %)	4 (8.9 %)	0 (0.0 %)		

cancer cells at the invasive fronts. Since no significant difference was observed between both assessments, we used the assessment of overall tumor tissues for further analyses.

The differential expression of claudin-1 and claudin-4 was shown in Table 2. The claudin-1 immunoreactivity was observed in 86.7 % of cases. The majority of them showed less than 25 % of positive cancer cells (level 1+; 33.3 %), followed by between 26 and 50 % of positive cells (level 2+; 26.6 %), more than 75 % of positive cells (level 4+; 20.0 %) and between 51 and 75 % of positive cells (level 3+; 6.7 %). The positive immunoreactivity of claudin-4 appeared to be less frequent (80.0 %) than that of claudin-1. More than 60 % of cases showed claudin-4 positivity in less than 25 % of cancer cells (level 1+), followed by between 51 and 75 % of positive cells (level 3+; 8.9 %) and between 26 and 50 % of positive cells (level 2+; 4.4 %), respectively. No case showed claudin-4 staining in more than 75 % of cancer cells (Fig. 1).

In addition, the correlation between the expression levels of claudin-1 and claudin-4 was examined. The statistically significant positive relationship was noted between the expression patterns of both proteins ($P = 0.03$) (Table 2).

Relationships Between the Claudin-1 and Claudin-4 Expression and the Clinico-Pathologic Features

For the analyses of claudin expression, cases were divided into 2 groups, the low expression group (cases with less than 50 % of positive cancer cells) and the high expression group (cases with more than 50 % of positive cancer cells). No sex or age difference was observed between the two groups.

Results are shown in Table 3. Significantly, the increased claudin-1 expression was associated with high pathologic grade ($P = 0.02$), high T stage ($P = 0.01$), the presence of microscopic perineural ($P = 0.03$) and

Table 3 Relationship between claudin-1 and claudin-4 expression and clinico-pathologic features of OSCC patients

Clinico-pathologic parameter	Claudin-1 expression			Claudin-4 expression		
	Low	High	*P* value	Low	High	*P* value
Sex						
Male	18	4	0.21	20	2	1
Female	15	8		21	2	
Age (years)						
<65	17	4	0.28	19	2	0.89
>65	16	8		22	2	
T stage						
T1–3	32	8	0.01	37	3	0.39
T4	1	4		4	1	
Lymph node involvement						
Absent	19	2	0.02	20	1	0.61
Present	14	10		21	3	
Distant metastasis						
Absent	32	11	0.47	40	3	0.172
Present	1	1		1	1	
Recurrence						
Absent	25	10	0.71	32	3	1
Present	8	2		9	1	
Pathologic grade						
Well-differentiated	21	3	0.02	23	1	0.33
Moderate/poorly differentiated	12	9		18	3	
Perineural invasion						
Absent	24	4	0.03	27	1	0.14
Present	9	8		14	3	
Vascular invasion						
Absent	22	4	0.04	23	3	0.63
Present	11	8		18	1	
TNM staging						
Stage I, II	23	1	0	23	1	0.33
Stage III, IV	10	11		18	3	

vascular ($P = 0.04$) invasions, regional lymph node involvement ($P = 0.02$) and advanced TNM stage ($P = 0.00$). On the contrary, no statistically significant relationship was noted between the claudin-4 expression and all clinico-pathologic features examined.

Survival Analysis

The follow up period ranged from 8 to 119 months (median = 38 months). At the end of the follow-up period, seventeen patients died of OSCC, 2 patients died of other causes and the remaining 26 patients were alive with no disease. The advanced clinical stage was strongly correlated with poor overall patient survival ($P = 0.01$). The univariate survival analysis showed a tendency towards the association of the higher claudin-1 expression and a shorter survival time (Fig. 2), however, this did not reach a statistically significant level ($P = 0.07$). Claudin-4 expression showed no statistically significant association with cancer-specific survival of patients ($P = 0.85$).

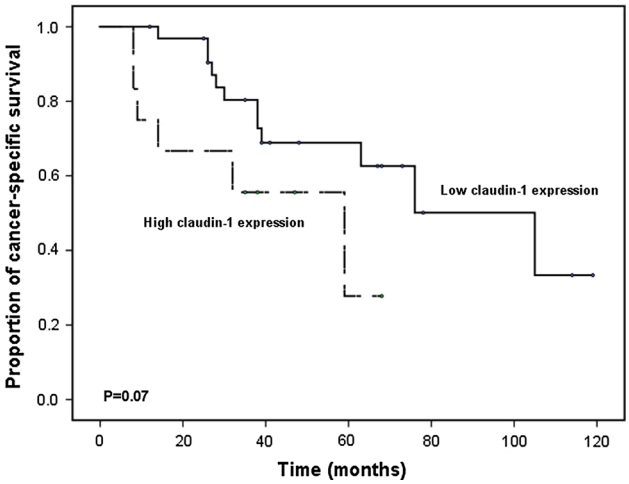

Fig. 2 Kaplan–Meier curve of OSCC patients with low (less than 50 % positivity) versus high (more than 50 % positivity) expression of claudin-1 ($P = 0.07$)

Discussion

An increasing number of studies have demonstrated the changes in the expression of different claudins in a variety of human cancers. Claudin-1 is perhaps the most studied protein among claudin family members. The reduced expression of claudin-1 was observed in breast and prostate cancers [10–12], however, a greater number of other cancers including gastric, thyroid, pancreatic, urothelial and cervical cancers instead showed increased claudin-1 expression [13–16]. This suggests that this protein may have tissue-specific functions and its roles in various cancers may be different depending on the type of cancer cells and/or the nearby cancer environment.

In this study, we reported the significance of differential expression of claudin-1 in OSCC. The overexpression of claudin-1 was observed in the more advanced diseases and associated with the aggressive histopathologic features, including perineural and vascular invasion. This indicates that claudin-1 may be either directly or indirectly involved in the progression of this cancer.

The underlying mechanisms of claudin-1 in the progression of several cancers are not completely understood and have become the basis of recent molecular studies. To date, no claudin gene mutation has been reported. Instead, recent evidence suggested that claudins may be involved in cancer progression through the complex interaction with

several extracellular matrix elements. In the expression cloning study, claudin-1 was shown to increase matrix metalloproteinase-2 (MMP-2) activity via its interaction with membrane-type matrix metalloproteinase-1 (MT1-MMPs) and enrich the localization of MMP-2 on the cell surface [35]. This could enhance the invasive potential of cancer cells through the degradation of surrounding extracellular matrix components, including the basement membrane. In colon carcinomas, claudin-1 upregulation was associated with increased cancer cell migration and MMP-2 and MMP-9 activities. In contrast, inhibition of claudin-1 in colonic cancer cells decreased their invasive/metastatic potential, promoted apoptosis and reduced cell survival [32].

A handful of studies reported the altered claudin-1 expression in OSCC [25, 31, 33]. Compared to the normal oral mucosa, claudin-1 expression was altered in different grades of oral epithelial dysplasia and squamous cell carcinoma [26, 31]. In contrast to the tissue microarray study by Lourenco et al. [31] which reported low-to-absent claudin-1 expression in moderately/poorly differentiated OSCCs, we found that increased claudin-1 expression is significantly associated with higher pathologic grade. This discrepancy of results may partly be related to tissue sampling error from the tissue microarray technique. In the present study, the entire tissue specimen from each case was analyzed and some variations of claudin staining in different areas of the section were noted. Therefore, sampling a selected portion of the specimens may not be entirely representative of overall lesions. In addition, the disagreement may also be a result of the differences in the methods of detection and the types of antibodies used with varying cross-reactivity potential. Nonetheless, in conjunction with our finding, Dos Reis et al. [33] found that the increased claudin-1 gene expression was associated with the increased angiolymphatic and perineural invasions, the prognostically relevant histopathologic features of OSCC [2].

A study of SCC of the lower lip revealed that the claudin-1 expression was higher in metastatic and advance-staged cases [27]. This finding is consistent with our results, even though in our study all lesions originated from intraoral sites. The pathogenesis of OSCC of the lower lip is considered different from the intraoral ones, due to some differences in the predisposing factors involved, such as sunlight. This suggests that claudin-1 may be involved in one of the common pathways in the development of head and neck SCC.

A molecular study that examined the role of claudin-1 in OSCC also pointed towards the interplay between cancer cells and extracellular matrix components. Oku et al. [34] found that the inhibition of claudin-1 expression in OSCC cell lines diminished cancer cell invasion and reduced degradation of laminin-5, an important component of the basement membrane, through MMP-2 and MT1-MMP inactivation. Overall, it appears that claudin-1 could be a potential marker of the advanced stage and aggressive histopathologic features of OSCC. The high expression of this protein is related to the more progressive lesions and consequently poor clinical outcome of patients.

Interestingly, while most of our cases showed the intense claudin-1 staining in the center of the tumor nests, some cases also contained positive cancer cells at the periphery in close proximity to the surrounding connective tissue. In addition, three cases demonstrated the higher percentage of claudin-1 positive cells at the invasive front, compared to that of the entire specimens. It will be of interest to examine in a larger case study whether these differential distribution of claudin-1 expression impacts the overall disease behavior and patient prognosis.

A number of studies reported the overexpression of claudin-4 in a variety of cancers [12, 14, 17–23]. The claudin-4 up-regulation was shown to stimulate MMP-2 activity in ovarian carcinoma cells and promote cancer invasion [36]. A strong correlation of claudin-4 expression and poor patient survival was also reported in a few cancers, such as gastric adenocarcinoma and endometrial carcinoma [15, 37]. In addition, based on a recent gene expression profiling study, claudin-4 was found to be a predictive marker for the poor response to radiation therapy of patients with head and neck SCC [38]. However, we did not find any significant relationships between the claudin-4 expression and patient clinical-pathologic features or survival data. Therefore, our data do not support the prognostic role of altered claudin-4 in patients with OSCC.

In conclusion, the present study demonstrates the relationship of differential claudin-1 expression in OSCC and selected clinical and pathologic parameters. The high claudin-1 expression in cancer cells is significantly associated with high pathologic grade, increased perineural/vascular invasion, increased propensity of lymph node metastasis and advanced clinical stage of tumor. These results suggest that claudin-1 may play a role in the progression of OSCC. Notably, the claudin-1 expression assessed immunohistochemically may be a potential indicator of advanced diseases in these patients.

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