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Intracellular claudin-1 at the invasive front of tongue squamous cell carcinoma is associated with lymph node metastasis

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Abstract

Claudins are the major component of tight junctions, which form a primary barrier to paracellular diffusion and maintain cell polarity in normal epithelia and endothelia. In cancer cells, claudins play additional roles besides serving as components of the tight junctions, and participate in anoikis or invasion. Among the claudin family proteins, claudin-1 has the most promising potential, both diagnostically and prognostically, in many types of cancers, including oral, gastric, liver, and colon cancers. However, conflicting results have been reported in relation to the degree of claudin-1 expression and the prognosis, suggesting that the expression level of claudin-1 alone is not sufficient to analyze the relationship between claudin-1 and cancer progression. As endocytic trafficking of claudin-1 has been reported in several epithelial cell types in vitro, we aimed to determine whether intracellular localization of claudin-1 is the missing aspect between claudin-1 and cancer. We investigated the expression of claudin-1 in 83 tongue squamous cell carcinoma (TSCC) pathological specimens. Although the expression level of claudin-1 based on immunohistochemistry was not associated with TSCC progression, within the high claudin-1 expression group, the incidence of intracellular localization of claudin-1 was correlated with cervical lymph node metastasis. In an in vitro experiment, claudin-1 was constitutively internalized in TSCC-derived cells. Motility of TSCC-derived cells was increased by deficiency of claudin-1, suggesting that the decrease in cell-surface claudin-1 promoted the cell migration. Therefore, intracellular localization of claudin-1 at the invasion front may represent a promising diagnostic marker of TSCC.

KEYWORDS

claudin-1, endocytosis, invasion front, metastasis, tongue squamous cell carcinoma

Abbreviations: CPZ, chlorpromazine; IMP, imipramine; OSCC, oral squamous cell carcinomas; TSCC, tongue squamous cell carcinoma.

1 | INTRODUCTION

The oral cavity is the major site of head and neck cancer,[1] with approximately 300 000 cases and 145 000 deaths reported annually worldwide.[2] The primary sites of cancer within the oral cavity are the tongue and the floor of the mouth, or the buccal mucosa. Surgical removal is followed by postoperative declines in speech and feeding, causing a serious decline in quality of life (QOL). Histologically, most oral cancers are squamous cell carcinomas that infiltrate into mesenchymal tissues. The important factor affecting the survival prognosis is metastasis.

Claudin-1 belongs to the claudin superfamily of proteins, which are structural and functional components of tight junctions.[3] Claudin-1 is classified as a paracellular barrier-forming claudin. Mice deficient in claudin-1 die of dehydration within a day of birth, indicating that claudin-1 in skin epidermal sheets is crucial for organizing the water barrier on the entire surface of the body.[4] Recent studies have suggested that claudin-1 also plays a role unrelated to tight junctions in cancer, such as the activation of tumor necrosis factor (TNF)-α[5] or c-Abl-ERK signaling,[5,6] the resistance to anoikis in a Src-dependent method[7] and the enhancement of invasive activity through the activation of matrix metalloproteinases.[8]

Low claudin-1 expression is associated with shorter overall survival and higher tumor grades in lung adenocarcinoma,[9] prostatic adenocarcinoma,[10] rectal cancer,[11] and breast cancer.[12-14] In these cancers, claudin-1 is a metastasis suppressor. In contrast, in colon cancer,[15] some studies have shown that claudin-1 overexpression has a good prognostic value (fewer metastases and less aggressive),[11,16,17] whereas other studies have shown that claudin-1 overexpression is linked with increased invasion.[15,18,19] Accordingly, although claudin-1 has the potential to be a biomarker with diagnostic and prognostic values in oral, gastric, liver, and colon cancers,[15] the seemingly contradictory results imply that the expression level of claudin-1 alone is not sufficient to analyze the relationship between claudin-1 and cancer metastasis.

The tight junction protein complex reportedly has a dynamic structure, undergoing rapid and continuous molecular remodeling in the steady state in MDCK cells.[20] A functional endosomal sorting complex required for transport (ESCRT) is involved in the constitutive recycling of claudin-1 between the plasma membrane and intracellular vesicles in MDCK cells.[21] Furthermore, there is growing evidence linking endocytosis and the regulation of cell migration.[22,23] Thus, we hypothesized that a factor related to the membrane dynamics of claudin-1 would be equivalent to the missing link between claudin-1 and cancer metastasis.

Cancer cell morphology at the invasion front has recently attracted attention as a prognostic factor. For example, in colon cancer, the correlation between tumor budding at the invasion front of the lesion and regional lymph node metastasis has been reported.[24] In oral cancer, the YK classification based on the infiltration mode has been used as one of the diagnostic criteria.[25]

Overexpression of the claudin-1 (*CLDN1*) gene is significantly correlated with advanced disease stage in OSCC based on molecular classifications of oral cancer by cDNA microarrays.[26] In line with this result, overexpression of the claudin-1 protein is associated with invasion in OSCC.[8,27]

In the present study, we investigated whether the intracellular location of claudin-1 at the invasion front of the lesion correlates with the degree of disease progression in OSCC. When the tongue is the primary lesion, the invasion front primarily resides in soft tissue, such as muscle tissue. In contrast, when the gingiva and the floor of the mouth are the primary lesions, the invasion front infiltrates the hard tissue, and thus a decalcification process is required prior to immunohistochemical staining, which may cause variations in the results. Therefore, we concentrated on TSCC, which represents the majority of OSCC and has the advantage that the decalcification process is unnecessary. We found that, within the high claudin-1 expression group, the incidence of intracellular claudin-1 localization was correlated with metastasis. Furthermore, constitutive endocytosis of claudin-1 was shown in the TSCC-derived cell line. Endocytosis of claudin-1 and cell motility was hindered by endocytosis inhibitors. In contrast, deficiency of claudin-1 increased cell motility. The present study suggested that the ectopic, intracellular location of claudin-1 at the invasion front may be an additional and promising diagnostic marker of TSCC.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

Primary tongue squamous cell carcinoma specimens were collected from 83 patients who had been treated at the Dental Hospital of Tokyo Medical and Dental University. All of the tissues were fixed with 10% neutral buffered formalin and embedded in paraffin according to routine laboratory protocols. All experimental procedures were conducted in accordance with the amended Declaration of Helsinki and approved by the ethics committee of Tokyo Medical and Dental University (registry no. D2015-534). We used surgically resected, formalin-fixed, and paraffin-embedded human TSCC tissues for the immunohistochemical study.

2.2 | Immunohistochemistry

For immunohistochemical staining, formalin-fixed, paraffin-embedded human TSCC tissue sections were used. Primary antibody was anti-claudin-1, rabbit polyclonal, 1:100 (ab15098; Abcam). Antigen retrieval was carried out according to the manufacturer's protocol. EnVision + Dual Link (Dako) was used as the secondary antibody, and coloration was conducted with the diaminobenzidine substrate.

2.3 | Evaluation of immunohistochemical analyses

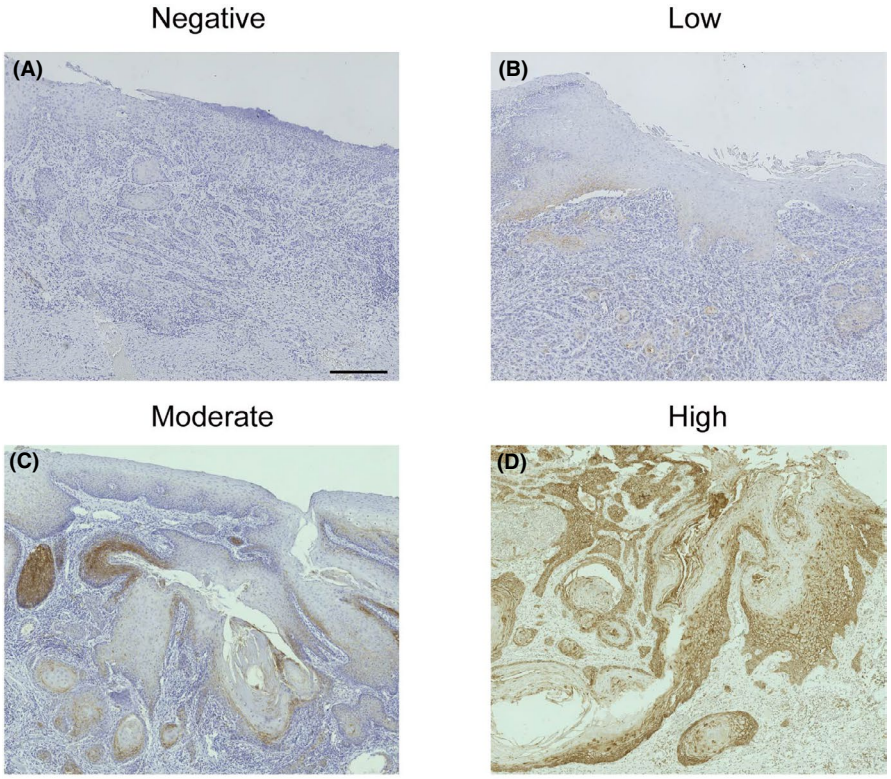
Immunohistochemical assessment was carried out and agreed upon by two pathologists who were blinded to all patient clinical and follow-up

data. Percentages of claudin-1-positive neoplastic cells were semiquantitatively assessed, and proportion scores of 1, 2, 3, and 4 were given when the percentages of positive cells were 25% or less, 26%-50%, 51%-75% and 76%-100%, respectively. Intensities of immunoreactivity were categorized into negative (score 0), low (score 1), moderate (score 2), and high (score 3) as shown in Figure 1. The final score of claudin-1 immunoreactivity levels was obtained by multiplying the proportion score by the intensity score. Cases were classified into high if the scores were 4 or higher and into low if the scores were 3 or less.

For assessment of the localization of claudin-1, five square regions were selected from the claudin-1-positive invasion front for each case. Cases were classified as "intracellular" when the exclusive membrane localization of claudin-1 was not observed in three or more regions. Otherwise, they were classified as "membrane".

2.4 | Confocal laser scanning microscopy

For immunofluorescence staining, a mouse monoclonal anti-claudin-1 antibody (1C5-D9)(H00009076-M01; Novus Biologicals) and an Alexa Fluor 488-conjugated anti-mouse antibody were used. Immunofluorescent images were captured and analyzed with a Leica TCS SP8 confocal microscope (Leica).



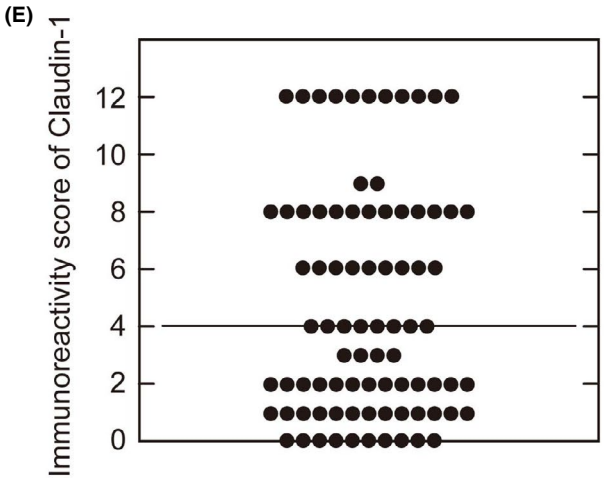


FIGURE 1 Representative photomicrographs of claudin-1 immunohistochemistry in tongue squamous cell carcinoma (TSCC). A-D, Immunoreactivity intensities were categorized into negative (score 0), low (score 1), moderate (score 2), and high (score 3). Scale bars, 300 μm. E, Distribution of the final scores of claudin-1 immunoreactivity (dimensionless) in TSCC (n = 83) obtained by multiplying the proportion score by the intensity score. Horizontal line shows the median value. Cases were classified into high- and low-expression groups, with final scores of 4 or more and 3 or less, respectively

TABLE 1 Clinicopathological features of 83 tongue squamous cell carcinoma patients

	No. of patients	(%)
Gender		
Male	50	
Female	33	
Age (y)		
Median	65.36	
SD	15.41	
T stage		
Carcinoma in situ (cis)	5	6.0
T1	43	51.8
T2	25	30.1
T3	9	10.8
T4a	1	1.2
T4b	0	0.0
TNM stage		
I	43	51.8
II	18	21.7
III	5	6.0
IVa	3	3.6
IVb	9	10.8
IVc	0	0.0
YK classification		
YK -1	2	2.4
YK-2	10	12.0
YK-3	35	42.2
YK-4c	25	30.1
YK-4d	6	7.2
Differentiation		
Well	42	50.6
Moderate	29	34.9
Poor	7	8.4
Local recurrence	7	8.4
Cervical lymph node metastasis	27	32.5
Distant organ metastasis	3	3.6

TABLE 2 Relationship between claudin-1 expression and clinicopathological features of 83 tongue squamous cell carcinoma patients

	No. of patients		
	Claudin-1 expression		
	High	Low	
Gender			
Male	24	26	
Female	19	14	$P = .52$
Age (y)			
≦69	20	15	
>69	23	25	$P = .54$
T stage			
T1	24	19	
T2-4	18	17	$P = .87$
TNM stage			
I	24	19	
I-IV	18	17	$P = .87$
Differentiation			
Well	24	18	
Moderate	14	15	
Poor	4	3	$P = .79$
YK classification			
YK -1	0	2	
YK-2	3	7	
YK-3	26	9	
YK-4c	10	15	
YK-4d	3	3	$P = .008$
Local recurrence			
Positive	5	2	
Negative	38	38	$P = .43$
Cervical lymph node metastasis			
Positive	17	10	
Negative	26	30	$P = .23$
Distant organ metastasis			
Positive	2	1	
Negative	41	39	$P = .00$

2.5 | Cell culture and reagents

Human tongue squamous cell carcinoma cell line (SAS) was obtained from Japanese Collection of Research Bioresources. SAS cells were maintained in DMEM: high glucose (08458; Nacalai Tesque), supplemented with 10% FBS (Sigma-Aldrich), L-penicillin (50 U/mL), and streptomycin (50 μg/mL), at 37°C in a 5% CO_2 atmosphere. Chlorpromazine hydrochloride (C8138) was obtained from Sigma-Aldrich and imipramine hydrochloride (I0971) was from Tokyo Chemical Industry.

2.6 | Endocytosis of claudin-1

Claudin-1 endocytosis was evaluated based on the modified cell-surface assay using EZ-link sulfo-NHS-SS-biotin (21 331; Pierce), as described previously.[21,28] Materials recovered by Neutravidin beads (29 200; Pierce) were subjected to 12% SDS-PAGE under reducing conditions, and then transferred to a PVDF membrane. The membrane was incubated with 4% Block Ace (Yukijirushi) for 1 hour and then incubated with an anti-claudin-1, rabbit polyclonal

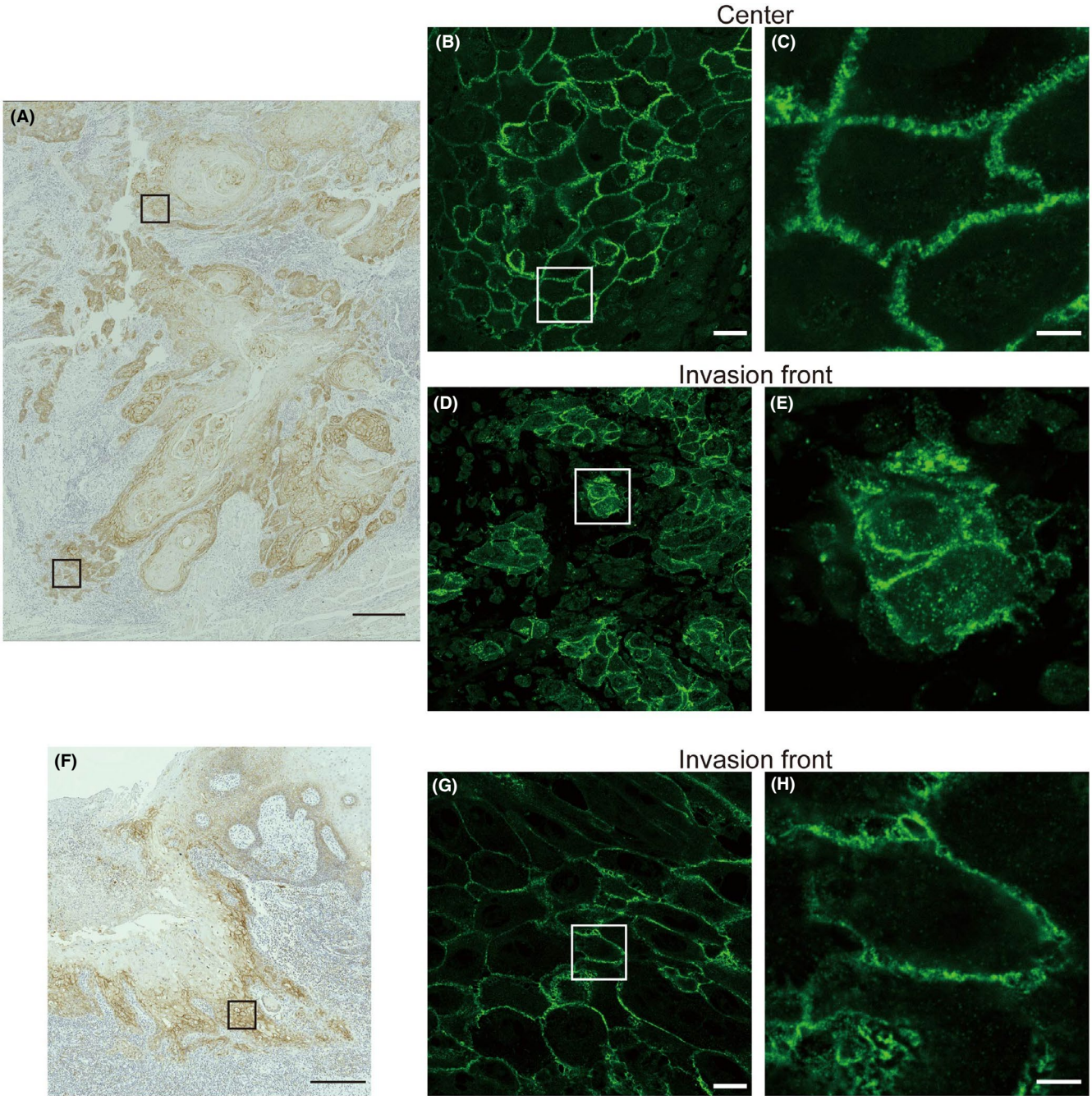


FIGURE 2 Typical cases of tongue squamous cell carcinoma (TSCC) showing either intracellular (A-E) or membrane-confined localization (F-H) of claudin-1 at the invasion front. Immunohistochemical (A, F) and immunofluorescence (B-E, G, H) staining of claudin-1 in two cases of TSCC. Upper and lower boxed areas in (A) show the center and the invasion front of the lesion, which are magnified in (B) and (D), respectively. The boxed area in (F) shows the invasion front of the lesion in another TSCC, which is magnified in (G). The boxed areas in (B, D, G) are further magnified in (C, E, H), respectively. See also Videos S1-S3, which correspond to (C, E, H), respectively. Scale bars: 300 μm (A, F), 25 μm (B, D, G), and 5 μm (C, E, H)

antibody (ab15098; Abcam). An HRP-conjugated anti-rabbit antibody (#7074; Cell Signaling Technology) was used as the secondary antibody. Immunoblot signals of claudin-1 were detected using the ECL Western Blotting Detection Reagent (RPN2109; GE Healthcare) and quantified by the FUSION chemiluminescence imaging system (VILBER).

2.7 | Cell motility assay

SAS cells were seeded at a density of 2×10^4/well into 6-well plates, 48 hours before the experiment. Cell motility was monitored with a BZ-X800 time-lapse imaging system (KEYENCE). Images were analyzed using ImageJ software (National Institutes of Health), and cell

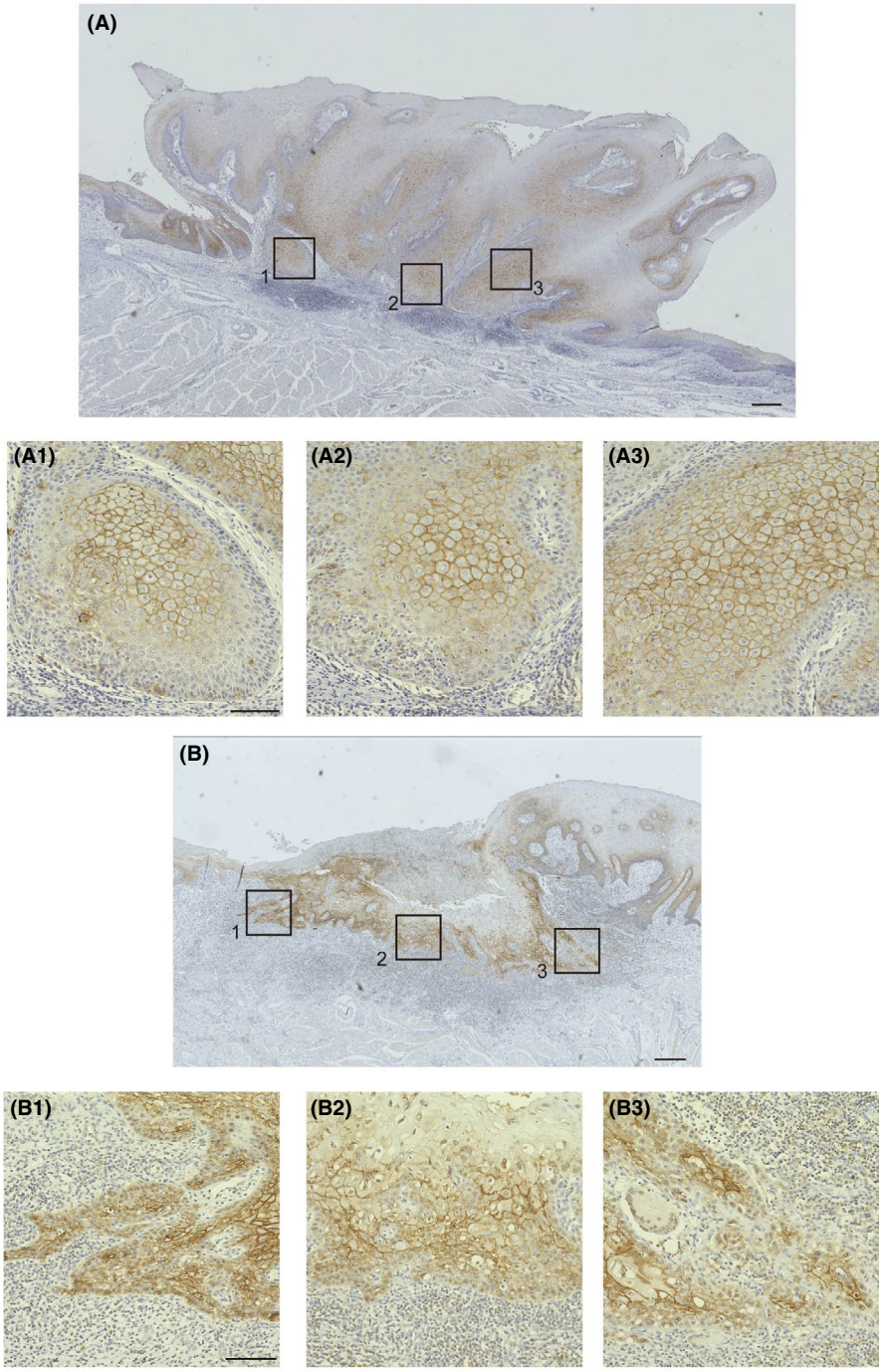


FIGURE 3 Assessment of tongue squamous cell carcinoma according to membrane localization of claudin-1 at the invasion front. For assessment of claudin-1 localization, five square regions were selected from the claudin-1 positive invasion front for each case. The case was classified as "membrane" when exclusive membrane localization of claudin-1 was observed based on immunohistochemical staining in more than three regions. This figure shows examples of membrane localization of claudin-1. Three boxed areas out of five in (A, B) are magnified (A-1/A-2/A-3, B-1/B-2/B-3), respectively, and show the membrane localization of claudin-1. Scale bars, 300 μm (A, B) and 100 μm (A-1/A-2/A-3, B-1/B-2/B-3). The case shown in Figure 3A is the same as that in Figure 2F-H

tracking was done using the Manual Tracking plugin. Total distance traveled was determined by tracking the movement of the cell gravity center, and its coordinates were used to calculate the distances.

2.8 | *Establishment of SAS cells deficient for the* claudin-1 *gene*

Claudin-1-knockout SAS cells were established using the clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/CAS9)-mediated genome editing system.[29] Guide RNA (gRNA) target sequences for claudin-1: 5′-CCTATGCCGGCGACAACATCGTG-3′ (gRNA #1) and 5′-CCTGCGTGTCGCAGAGCACCGGG-3′ (gRNA #2) (PAM sequence underlined) were designed with CRISPRdirect (https://crispr.dbcls.jp). Expression cassette of each gRNA was incorporated into the pX330 plasmid, carrying the human Cas9 expression cassette. Efficiency of both gRNAs to induce double strand breaks was validated by reconstituted EGFP fluorescence, as described previously.[30] After transfection of SAS cells with the pX330 plasmid, knockout clones were isolated by carrying out limiting dilution. Effective knockout of claudin-1 was confirmed at the protein

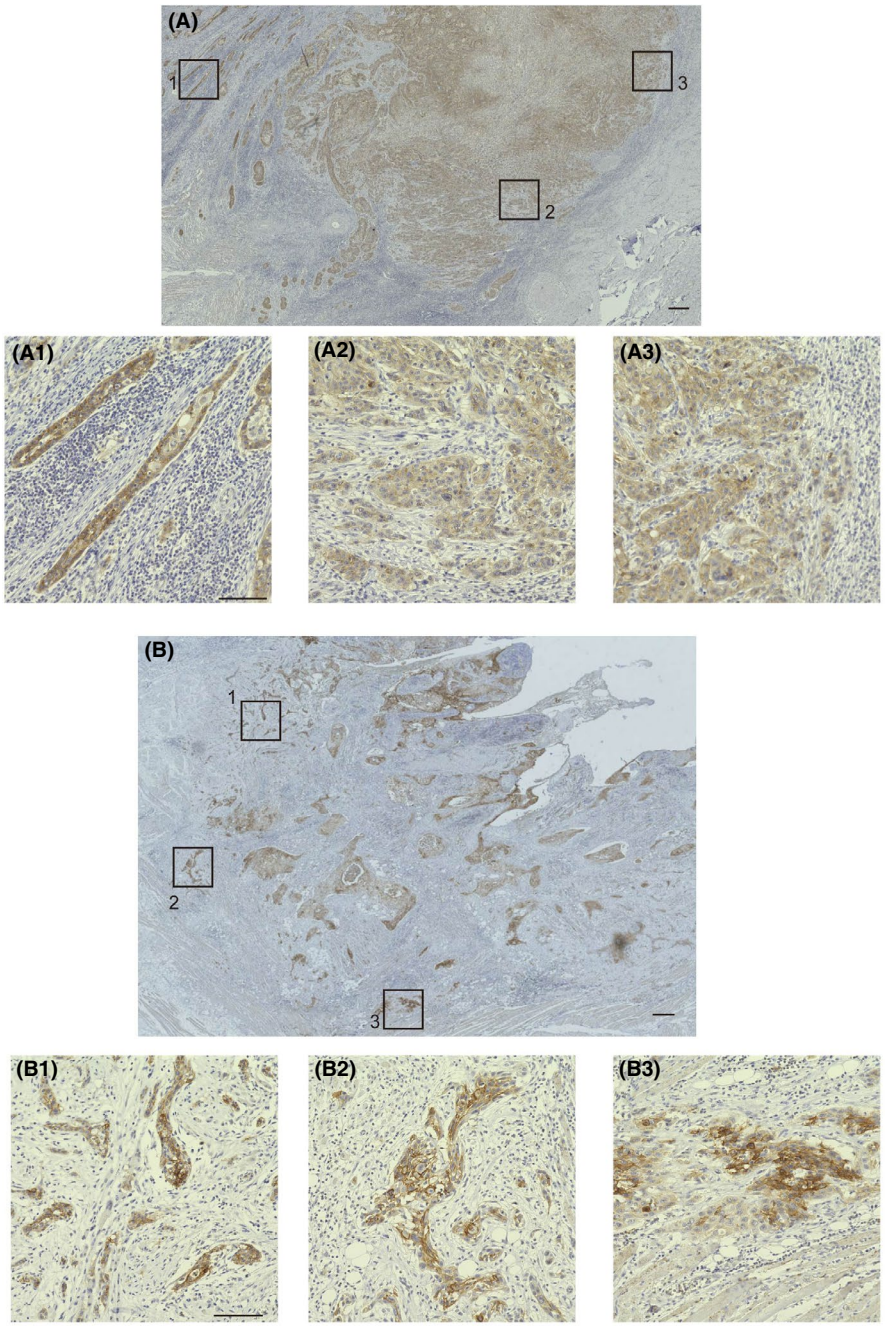


FIGURE 4 Assessment of tongue squamous cell carcinoma according to intracellular localization of claudin-1 at the invasion front. This figure shows examples of the intracellular localization of claudin-1. Three boxed areas out of five in (A, B) are magnified in (A-1/A-2/A-3, B-1/B-2/B-3), respectively, and show the intracellular localization of claudin-1. Scale bars, 300 μm (A, B) and 100 μm (A-1/A-2/A-3, B-1/B-2/B-3)

level by immunoblotting with an anti-human claudin-1 antibody (ab15098).

2.9 | Statistical analysis

In clinical specimens, Fisher's exact test or Pearson's chi-squared test were carried out with the statistical software 'EZR' (Easy R).[31] For the in vitro experiments, significant differences between means were determined using Student's *t* test. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Claudin-1 expression level is not associated with TSCC progression

Paraffin-embedded sections of TSCC specimens from 83 individual patients were examined for claudin-1 immunoreactivity. Patient details are provided in Table 1. Various degrees of claudin-1 immunoreactivities were observed within the basal layers of the epithelium and metastatic lesions as shown in Figure 1. Claudin-1 expression was not observed in the surrounding mesenchymal

TABLE 3 Relationship between localization of claudin-1 expression and clinicopathological features of claudin-1-high patients

	No. of patients		
	Intracellular	Membrane	
Differentiation			
Well	9	15	
Moderate	8	6	
Poor	4	0	$P = .06$
YK classification			
YK-1	0	0	
YK-2	0	3	
YK-3	12	14	
YK-4c	6	4	
YK-4d	3	0	$P = .10$
Local recurrence			
Positive	4	1	
Negative	17	20	$P = .34$
Cervical lymph node metastasis			
Positive	15	2	
Negative	6	19	$P = .0000929$
Distant organ metastasis			
Positive	2	0	
Negative	19	21	$P = .48$

tissue. All of the cases were classified into either the high or low expression group, based on both the intensity and proportion scores. Results are shown in Table 2. There were no significant differences in any of the evaluation items between the two groups.

3.2 | Intracellular localization of claudin-1 at the invasion front is associated with cervical lymph node metastasis

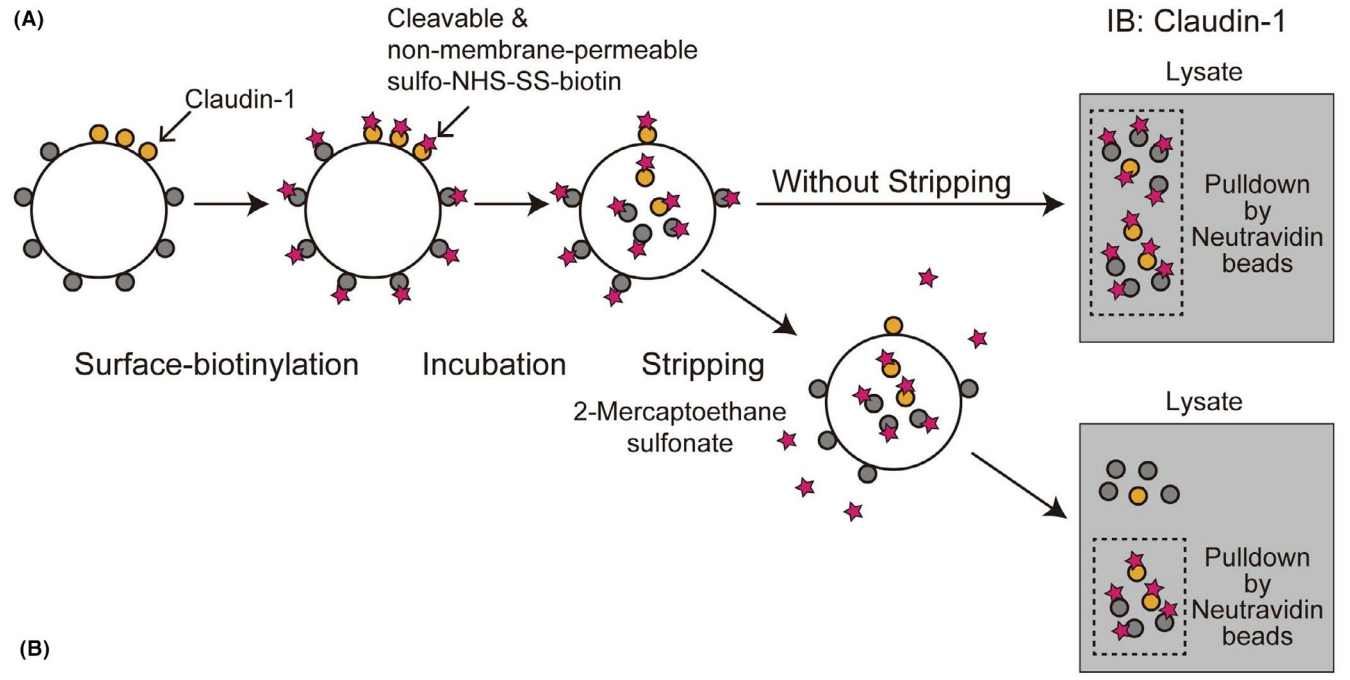
In some specimens, we noticed a difference in claudin-1 immunostaining between the center and the invasion front of the lesion, which may be due to an altered localization of claudin-1 (Figure 2A). Therefore, claudin-1 immunoreactivity was observed by immunofluorescence using confocal microscopy. Claudin-1 was predominantly localized at the membrane in the center of the lesion (Figure 2B,C), but intracellular claudin-1 localization was also observed at the invasion front (Figure 2D,E), as detected by 3-D images (Videos S1 and S2, respectively). Claudin-1 is likely localized to intracellular vesicles. Although nuclear localization of claudin-1 was reported in the case of colon cancer,[18] the nucleus was not the main site of localization in the present study. Although intracellular localization of claudin-1 was observed in some specimens, exclusive membrane localization was maintained at the invasion front in other specimens (Figure 2F-H and Video S3).

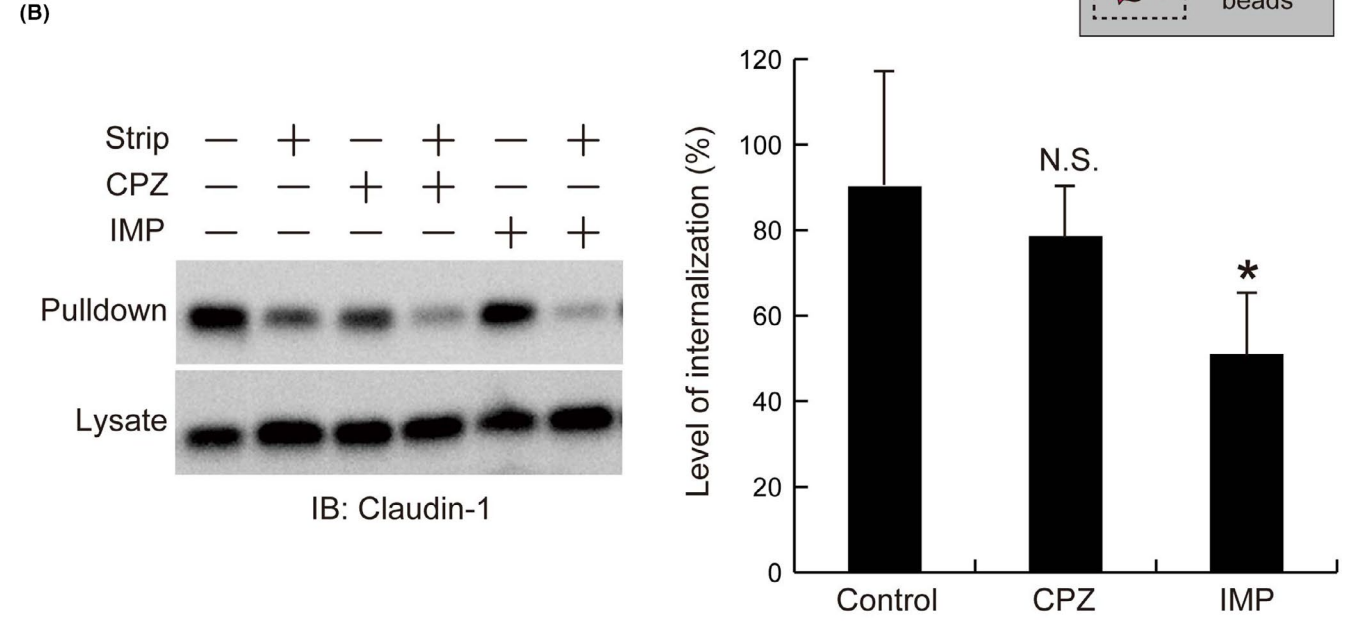
Thus, we developed a scoring system by which the localization of claudin-1 at the invasion front was evaluated as either "membrane" (Figure 3) or "intracellular" (Figure 4). Among the cases categorized as having high claudin-1 expression, the frequency of cervical lymph node metastasis was significantly higher in the "intracellular claudin-1" group than in the "membrane claudin-1" group, as shown in Table 3.

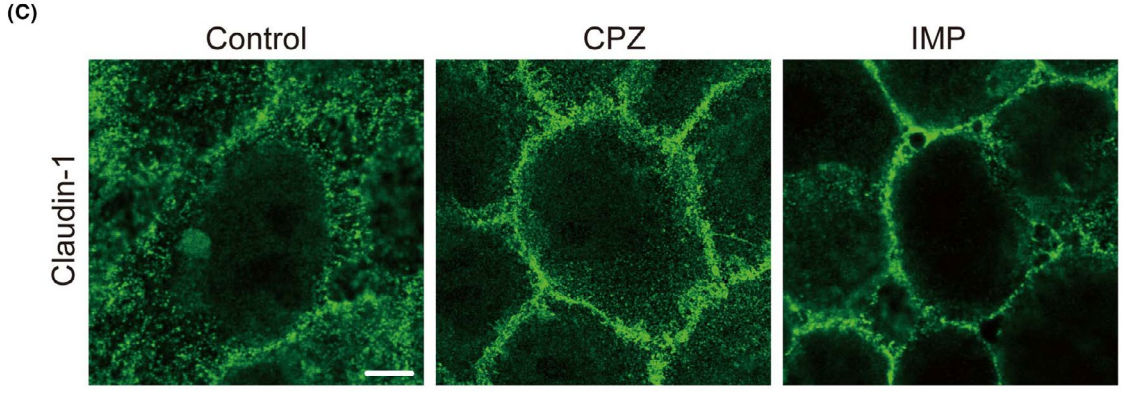
3.3 | Claudin-1 is constitutively endocytosed in TSCC-derived cells

SAS cells are derived from TSCC. Using these cells, we investigated the ability of claudin-1 to be endocytosed. For this purpose, cell-surface claudin-1 was biotinylated by a cleavable, cell-impermeable crosslinker (Figure 5A). After incubation, biotinylated residues remaining on the cell surface were either left or stripped off. The biotinylated claudin-1 was then recovered with Neutravidin beads and evaluated by immunoblotting. The biotinylated claudin-1 that remained after stripping was considered to be endocytosed from the cell surface. As shown in Figure 5A,B, a substantial part of the biotinylated claudin-1 was resistant to stripping, which is a biochemical demonstration of the endocytosis of claudin-1 in SAS cells. In addition, endocytosis of claudin-1 in SAS cells was investigated in the presence of an inhibitor of clathrin-dependent endocytosis, CPZ,[32] and an inhibitor of macropinocytosis, IMP.[33] Addition of IMP decreased the extent of stripping-resistant claudin-1, suggesting that IMP inhibited claudin-1 endocytosis. Although the addition of CPZ was also found to decrease the extent of stripping-resistant claudin-1, the effect was not statistically significant. However, immunofluorescence staining of claudin-1 in SAS cells

FIGURE 5 Claudin-1 is constitutively endocytosed in SAS cells. A, The protocol for evaluating the endocytosis of cell-surface proteins was described previously.[21,28] Cell-surface proteins were labeled by sulfo-NHS-SS-biotin. After incubation to allow the endocytosis of cell-surface molecules, the remaining biotin conjugated to the cell-surface molecules was either retained (without stripping) or cleaved by reduction (stripping). The cells were then lysed and the biotinylated materials were recovered by Neutravidin beads (Pierce) (pulldown). B, SAS cells were seeded in 35-mm dishes (2×10^5 cells/dish). The day after cells became confluent, the cells were subjected to surface-biotinylation assay for evaluation of endocytosis. During the incubation, the cells were treated without or with 10 μM chlorpromazine (CPZ) or 1 μM imipramine (IMP). After the amounts of Neutravidin-captured (pulldown) claudin-1 were normalized to their respective values in the lysates, level of internalization was expressed as the ratio between the values with and without stripping, as shown in the graph. Data are expressed as means + SD (n = 4). Student's *t* test with two biological independent replicates was used to determine statistical significance; $^*P < .05$; N.S., not significant compared with control. C, SAS cells were seeded on 15-mm round glass coverslips in 12-well plates (3×10^4 cells/well). After 72-96 h, cells were treated without or with 10 μM CPZ or 1 μM IMP for 2 h. The cells were fixed with 4% PFA for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and then labeled with an anti-claudin-1 antibody. Scale bars, 5 μm







revealed that the abundant intracellular staining of claudin-1 was suppressed in the presence of CPZ or IMP (Figure 5C). These results suggested that claudin-1 is constitutively endocytosed in TSCC cells. We considered that the loss of membrane location of claudin-1 is mainly caused by macropinocytosis with some contribution of clathrin-dependent endocytosis.

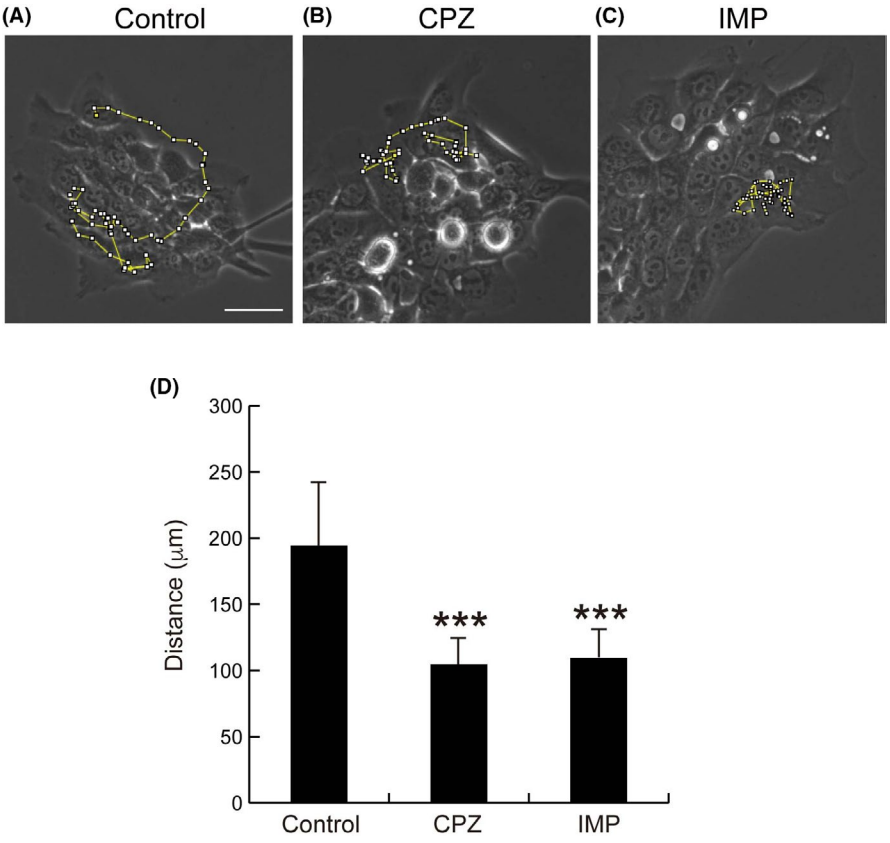


FIGURE 6 Endocytosis inhibitors chlorpromazine (CPZ) and imipramine (IMP) reduce the motility of SAS cells based on cell motility analysis. SAS cells were seeded in 6-well plates (2×10^4 cells/well). A-C, After 48 h, cells were incubated without or with 10 μM CPZ or 1 μM IMP, and cell motility was recorded. Representative random moving traces of 61 cells during a 10-min recoding period over 10 h are presented. Scale bar, 50 μm. D, Summarized data of the migrated distances within 10 h. Data are expressed as means + SD (n = 10). Student's *t* test with two biological independent replicates was used to determine statistical significance; ***$P < .001$, compared with control

3.4 | Inhibition of endocytosis suppresses the motility of SAS cells

The molecular machineries required for clathrin-dependent endocytosis are reportedly involved in the regulation of migration.[22,23,34] As constitutive endocytosis of claudin-1 was shown in SAS cells (Figure 5), we examined the effects of endocytosis inhibitors (CPZ and IMP) on the migration of SAS cells. For this purpose, we used the cell tracking method of recent studies.[35-37] As shown in Figure 6 (Videos S4-S6), total migrated distance was decreased by the addition of CPZ and IMP, suggesting that inhibition of endocytosis suppresses cell migration.

3.5 | CRISPR/CAS9-mediated knockout of claudin-1 in SAS cells accelerates cell motility

Finally, we investigated whether claudin-1 is involved in cell motility in SAS cells. For this purpose, claudin-1-knockout SAS cells were established by CRISPR/CAS9-mediated genome editing (Figure 7A). As shown in Figure 7B-E (Videos S7-S9, respectively), total migrated distance was increased in two independent clones of claudin-1-knockout SAS cells. These results suggested that claudin-1 has a great impact on the migration of SAS cells.

Both clathrin-dependent endocytosis and micropinocytosis[38] are involved in the regulation of cell migration. Thus, the possibility remained that the effects of CPZ and IMP (Figure 6) are independent of their actions on the endocytosis of claudin-1. However, considering that deficiency of claudin-1 promoted cell motility (Figure 7), it can be speculated that the disappearance of claudin-1 from the membrane by endocytosis promotes cell migration.

4 | DISCUSSION

Numerous epithelial-derived cancers show altered expression patterns of claudins.[39] In the present study, the expression level of claudin-1, as assessed by immunohistochemistry, was not associated with disease progression in 83 TSCC. However, within the high claudin-1 expression group, the loss of the exclusive membrane localization of claudin-1 (intracellular localization of claudin-1) at the invasion front was associated with cervical lymph node metastasis. This result suggested that the intracellular localization of claudin-1 at the invasion front could be a potential marker of metastasis in TSCC.

Previously, claudin-1 overexpression was reported to be associated with invasive pathological characteristics in OSCC in two independent studies which analyzed various regions in the oral cavity.[27,40] The areas in one study (total 99 patients) included the tongue (44%), floor of mouth (24%), floor of mouth and tongue (13%), alveoli (13%), buccal mucosa (5%), and retromolar trigone (1%).[27] Those of the other study (total 45 patients) included the gingiva (38%), tongue (16%), floor of mouth (18%), buccal mucosa (13%), hard palate (7%), and alveolar mucosa (9%).[40] In the

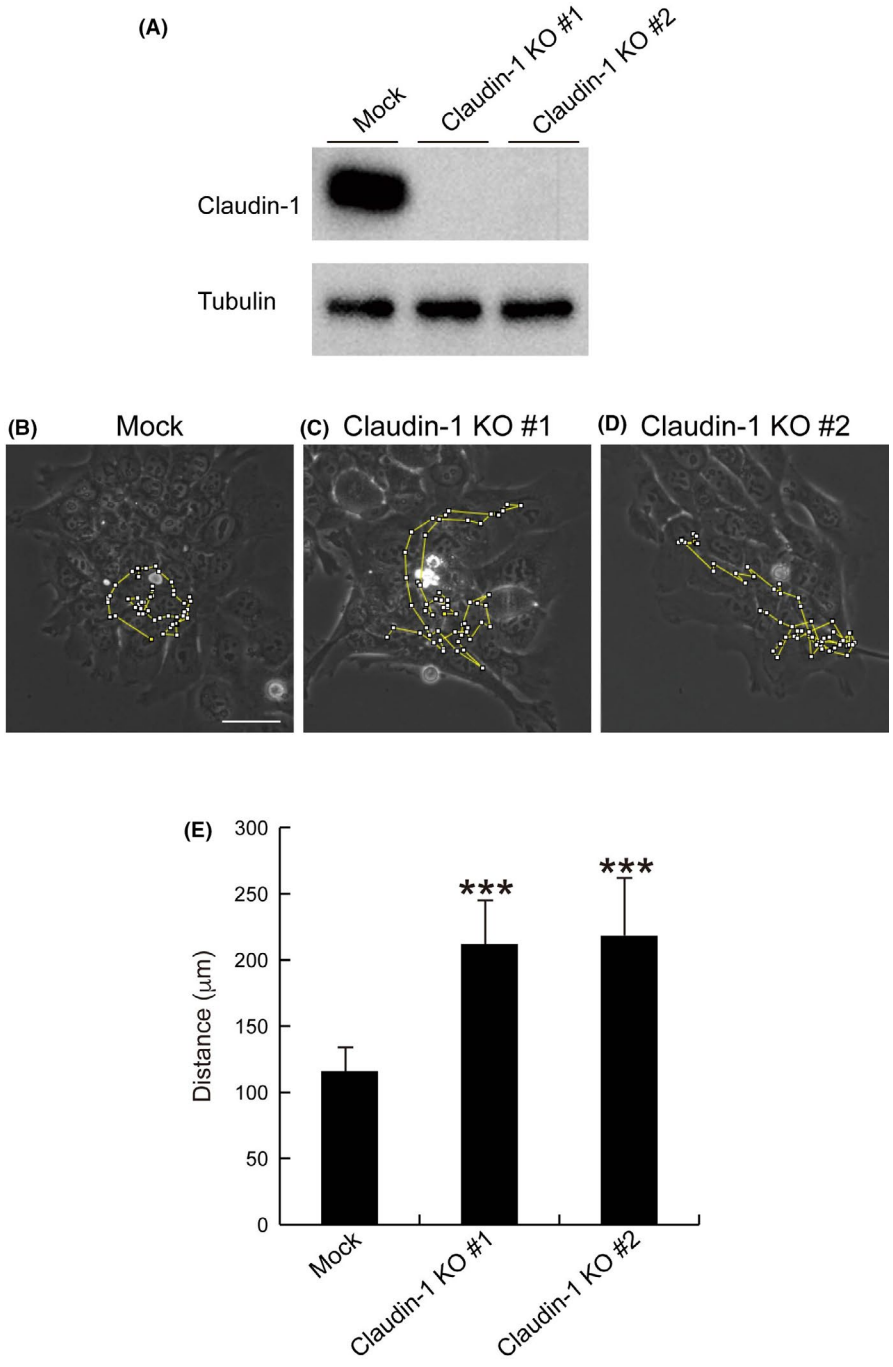


FIGURE 7 Deficiency of claudin-1 promotes motility of SAS cells based on cell motility analysis. A, Claudin-1 expression in mock or claudin-1-knockout SAS cells (generated by gRNA #1 and gRNA #2) was determined by immunoblotting. B-D, Mock or claudin-1-knockout SAS cells (generated by gRNA #1 and gRNA #2) were seeded in 6-well plates (2×10^4 cells/well). After 48 h, cell motility was recorded. Representative random moving traces of 61 cells during a 10-min recoding period over 10 h are presented. Scale bar, 50 μm. (E) Summarized data of the migrated distances within 10 h. Data are expressed as means + SD (n = 10). Student's *t* test with two biological independent replicates was used to determine statistical significance; ***$P < .001$, compared with control

present study, we focused on TSCC, which may explain the different conclusions obtained between the previous and present studies. As for our study, considering that the opposite conclusions of poor[18,19] and good[11,16,17] prognoses have been reported for high expression of claudin-1 in colon cancer,[15] it is possible that opposite types of TSCC coexist.

As the immunoreactivity of claudin-1 was restricted to the lesion in TSCC, claudin-1 in the cancer lesion may have a direct effect on invasion, rather than on the surrounding mesenchymal tissue. Expression of claudin-1 in cancer is regulated in various ways: claudin-1 is upregulated by β-catenin/Tcf signaling in human colorectal cancers,[19] and epigenetically silenced in estrogen receptor-positive breast cancer.[41] Thus, the expression level of claudin-1 in cancer cells could be context-dependent. During epithelial-mesenchymal transition (EMT), transcription factors Slug and Snail act as repressors of claudin-1, leading to reduction of the expression of claudin-1.[42] In contrast, claudin-1 reportedly promotes EMT through activation of the c-Abl/ERK signaling pathway[6] or facilitates invasion by disrupting interaction with the extracellular matrix through MMP.[8] In this context, high claudin-1 expression is predicted to promote invasion. In the present study, there was no relationship between the expression levels of claudin-1 and metastasis in TSCC patients (Table 2), suggesting that pathological TSCC belonging to "claudin-1-low" type and "claudin-1-high" type coexisted.

When we focused on the "claudin-1-high" type, the frequency of cervical lymph node metastasis was significantly higher in the "intracellular claudin-1" group than in the "membrane claudin-1" group (Table 3). The role of claudin-1 as a component of the tight junction may inhibit the invasion.[43] In SAS cells, endocytosis of claudin-1, as well as cell motility, was hindered by the endocytosis inhibitors (Figures 5 and 6). However, the deficiency of claudin-1 in SAS cells increased cell motility (Figure 7). Combining these results, it is possible that the endocytosis-mediated decrease in claudin-1 on the membrane promotes cell motility. Thus, we propose that the endocytosis of claudin-1 at the invasion front could relieve the effect of the persistence of tight junctions to inhibit cell motility in the "claudin-1-high" type.

We also noticed that membrane localization of claudin-1 at the invasion front tended to correlate with higher differentiation (Table 3). Cell polarity is an important aspect of cell differentiation. As claudin-1 is reportedly important in maintaining cell polarity,[21] we speculate that membrane localization of claudin-1 at the invasion front may contribute to maintaining the cell polarity and suppress dedifferentiation.

As tight junction proteins are overexpressed in several types of carcinomas, different approaches using monoclonal antibodies, enterotoxins, and therapeutic gene delivery have been tested as candidates for anticancer drug therapy.[43] The present study suggests that a specific inhibitor of the endocytosis of claudin-1 will be therapeutically valuable to suppress some cases of cancer metastasis, including TSCC.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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