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Claudins 1, 4, 5, 7 and occludin in ameloblastomas and developing human teeth

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BACKGROUND: To analyze the distribution pattern of claudins 1, 4, 5, 7 and occludin in benign and malignant ameloblastomas and developing human teeth.

METHODS: Paraffin-embedded tissue specimens of 25 benign and four malignant ameloblastomas and two developing human teeth were examined immunohistochemically using antibodies against claudins 1, 4, 5, 7 and occludin.

RESULTS: In ameloblastomas strongest expression was seen for claudins 1 and 7 while claudin 4 was expressed less frequently. Claudin 5 and occludin were seen only in a minority of cases. There were no evident differences in the expression of claudins or occludin neither between different histologic subtypes of ameloblastomas nor between benign or malignant cases. The strongest expression for claudins was present in the central stellatum reticulum-like cells surrounding the microcysts and in the areas with squamous differentiation of the ameloblastomas. In developing teeth both claudin 1 and 7 stained strongly in the enamel epithelium, ameloblasts, and enamel matrix, but staining for claudin 4 was relatively weak. Claudin 5 was preferentially expressed only in vessels, and occludin staining ranged from negative to weak in ameloblastomas and teeth germs.

CONCLUSION: There were no clear differences in the expression levels between benign and malignant ameloblastic tumors. The overexpression of claudins in the areas with microcyst formation may indicate their attempt to maintain the interepithelial cohesion of the cells. The strong immunoreactivity of ameloblasts and newly synthesized enamel matrix for claudins 1 and 7 indicates that they may be involved in cell signaling influencing enamel formation.

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Introduction

Structural integrity of tissues depends on intact barrier function of epithelia and endothelia which in turn is due to the presence and activity of intercellular tight junction (TJ) complexes. Tight junctions comprise three major transmembrane proteins, namely claudins, occludin, and junctional adhesion molecules (JAMs; 1, 2). TJs serve as physical barriers regulating transport of ions, water, and proteins between epithelial cells with site-dependent selectivity (gate function), in addition to playing the important role of maintaining cell polarity (fence function; 3). Recently, there are at least 24 known subtypes of claudins and three JAMs (4, 5). Claudins have a molecular weight of approximately 22 kDa [predicted to range between 20 and 27 kDa (6)], while occludin weighs 64 kDa (767 kDa). JAMs are predicted to weigh between 30 and 40 kDa from primary structure analysis (4).

Claudins are essential for the barrier function of epithelia and endothelia as they are thought to be responsible for the variety of electrical resistance and paracellular ionic selectivity seen in epithelia and endothelia (2, 4). The precise role of occludin in TJs is presently unknown although there is growing evidence that it is more important for cell signaling than for forming the paracellular barrier (8). Expression of claudins may vary in different cells and tissues of the body (9). For example, claudin 2 is found in murine liver and kidney but not in lung tissue while claudin 4 is found in murine lung and kidney but not in the liver (7). Variable expression of claudins has also been reported in rat liver and pancreatic cells (10).

Ameloblastomas are usually benign but locally invasive odontogenic tumors with a strong tendency to recur. Distant metastasis of ameloblastoma is a rare occurrence. They are the most common clinically

significant odontogenic tumors (11). The tumor constitutes approximately 1% of all cysts and tumors of the jaws (12). Three clinical types are described: solid, unicystic, and peripheral. The solid type can be subdivided histologically into follicular, plexiform, acanthomatous, desmoplastic, granular, and basal cell forms. The unicystic type can be classified into luminal, intraluminal, and intramural depending on the direction of the invasion of the tumor. Essentially the tumor consists of epithelial neoplastic islands or strands made up of peripheral columnar or cuboidal cells surrounding a central core of loosely arranged, angular cells. The peripheral cells resemble ameloblasts or pre-ameloblasts while the central cells are similar to the stellate reticulum cells of the dental germ.

Previously expression of claudins and occluding have been found in tumors derived from cells containing TJs, such as epithelial and endothelial tumors (13, 14). Individual claudins, however, may show selective expression, such as in vascular tumors which mainly express claudin 5 (13). In epithelial tumors, expression of claudins may also vary and loss or gain in their expression has been associated with biologic behavior in some tumor types (13, 15–18). As to the best of our knowledge, there are no studies on the expression of claudins and occludin in odontogenic tumors our aim was to analyze the staining patterns of claudins 1, 4, 5, 7 and occludin in benign and malignant ameloblastomas and developing human teeth.

Materials and methods

Samples

Twenty-nine cases of ameloblastomas and two dental germs were retrieved from the archives of the Departments of Diagnostics and Oral Medicine, University of Oulu and Pathology, Radboud University, Nijmegen, the Netherlands. For morphologic analysis, 5 μm sections were obtained from the paraffin-embedded samples and routinely stained with hematoxylin and eosin. The ameloblastomas were histologically classified into follicular ($n = 7$), plexiform ($n = 12$), acanthomatous ($n = 2$), mixed (being those with almost equal content of 2 or 3 histologic types; $n = 4$), and malignant (ameloblastic carcinoma; $n = 4$). The study was approved by The Ethical Committee of The Northern Ostrobothnia Hospital District.

Immunohistochemistry

The primary antibodies used in the immunostaining were all purchased from Zymed Laboratories (South San Francisco, CA, USA) designed to be used in formalin-fixed, paraffin-embedded tissues. They were polyclonal rabbit anticlaudin 1 (clone JAY.8), monoclonal mouse anticlaudin 4 (clone 3E2C1), monoclonal mouse anticlaudin 5 (clone 4C3C2) and polyclonal rabbit anticlaudin 7 (clone ZMD.241), and rabbit antioccludin. Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 min. For occludin, the section was treated with pronase (Tris-HCl, pH 7.6) for 8 min at 37°C and then cooled for 3 min at 4°C. After 60 min incubation with the primary antibody (dilution 1:50 for anticlaudins 1, 4 and 5, and 7), a biotinylated secondary antibody and Histostain-SP kit (Zymed Laboratories) was used. For all the immunostaining procedures, the color were developed by diaminobenzidine, after which, the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). The staining was carried out manually.

Negative control staining was carried out by substituting non-immune rabbit or mouse serum and PBS for the primary antibodies. As positive controls, non-neoplastic kidney, breast, skin, and liver samples were used.

The immunostaining was assessed as follows: −, no immunostaining present; +, <25% of cells positive; ++, 25–75% of cells positive; +++, more than 75% of cells positive. Both immunoreactivity associated with the cellular membrane and immunoreactivity associated with cytosol were used in the evaluation. The whole areas of the sections were screened.

In statistical analysis of the data, Fisher's exact test was used.

Results

Claudins 1, 4, 5, 7 and occludin in ameloblastomas

Claudin 1 showed strong immunoreactivity for central cells, especially in the areas of a squamous differentiation, of all ameloblastoma types analyzed (Figs 1a,b, 2a,b, 3a,b and 4e,f and Table 1). The peripheral cells were stained weakly or moderately predominating with the moderate staining (Figs 1b, 2b and 3b and Table 1). There were significantly more cases displaying strong staining in central than peripheral cells in benign ameloblastomas ($P = 0.049$), and also after assessing both benign and malignant ameloblastomas together ($P = 0.036$). In ameloblastic carcinomas, the immunoreactivity was strong in both central and peripheral cells with moderate immunostaining observed in half of the cases (Fig. 4a,e,f). No weak staining was observed (Table 1).

Claudin 4 was essentially negative in peripheral cells (Figs 1c, 2c, 3c and 4b and Table 1) but showed strong immunoreactivity in the central cells of acanthomatous ameloblastoma and ameloblastic carcinoma (Fig. 4c and Table 1). In the plexiform and mixed type, the immunoreactivity was weak in the central cells (Table 1). There were significantly more cases displaying strong staining in central than peripheral cells in benign ameloblastomas ($P = 0.047$), and also after assessing both benign and malignant ameloblastomas together ($P = 0.029$).

Claudin 5 was negative in most peripheral cells except in ameloblastic carcinomas in which weak immunostaining was observed in half the cases. The central cells demonstrated weak immunoreactivity in some cases (Figs 1d, 2d and 3d and Table 1). In one plexiform type strong immunostaining was observed in both cell types (Table 1). In addition, strong

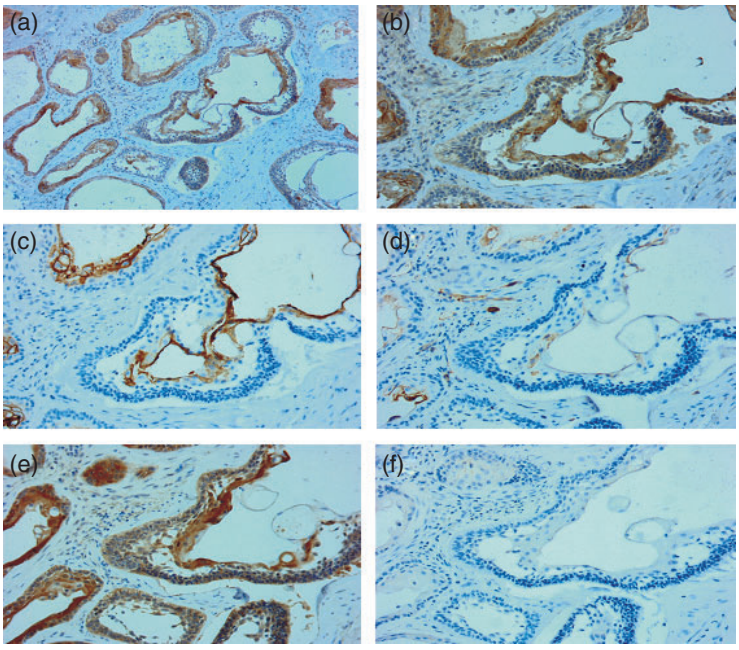

Figure 1 Immunohistochemical reactivity for claudins 1, 4, 5 and 7 and occludin in follicular ameloblastoma. (a and b) Strong immunoreactivity of the central cells and moderate immunoreactivity of the peripheral cells for claudin 1 (×50 and ×100 respectively). (c) Strong immunoreactivity for claudin 4 in the central cells, particularly in cells surrounding the cysts within the follicles, while the peripheral cells are negative (×100). (d) Weak immunoreactivity of central cells for claudin 5 (×100). (e) Strong immunoreactivity of central and peripheral cells for claudin 7 (×100). (f) Negative immunoreactivity for occludin (×100).

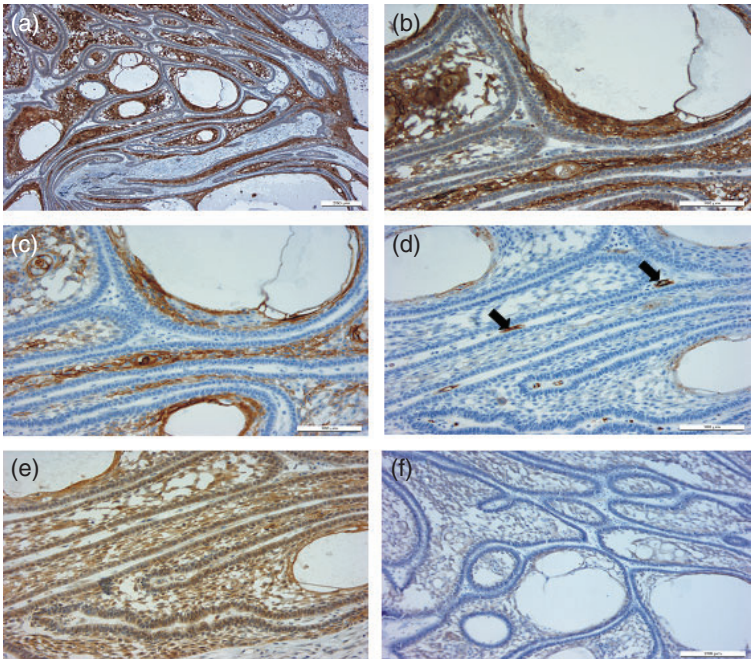

Figure 2 Immunohistochemical reactivity for claudins 1, 4, 5 and 7 and occludin in plexiform ameloblastoma. (a and b) Strong immunoreactivity for the central cells and weak immunoreactivity for the peripheral cells for claudin 1 (×50 and ×100 respectively). (c) Strong immunoreactivity for claudin 4 in central cell. Peripheral cells were negative (×100). (d) Weak immunoreactivity for central cells in claudin 5. Note the strong immunoreactivity for vascular structures (arrows) (×100). (e) Strong immunoreactivity for claudin 7 (×100). (f) Moderate immunoreactivity in central cells for occludin (×100).

immunostaining of vascular structures was observed in all the various types of ameloblastoma analyzed (Figs 1d, 2d, 3d and 4c).

Claudin 7 showed strong immunoreactivity for the central and peripheral cells of most benign ameloblastomas (Figs 1e, 2e and 3e and Table 1). However,

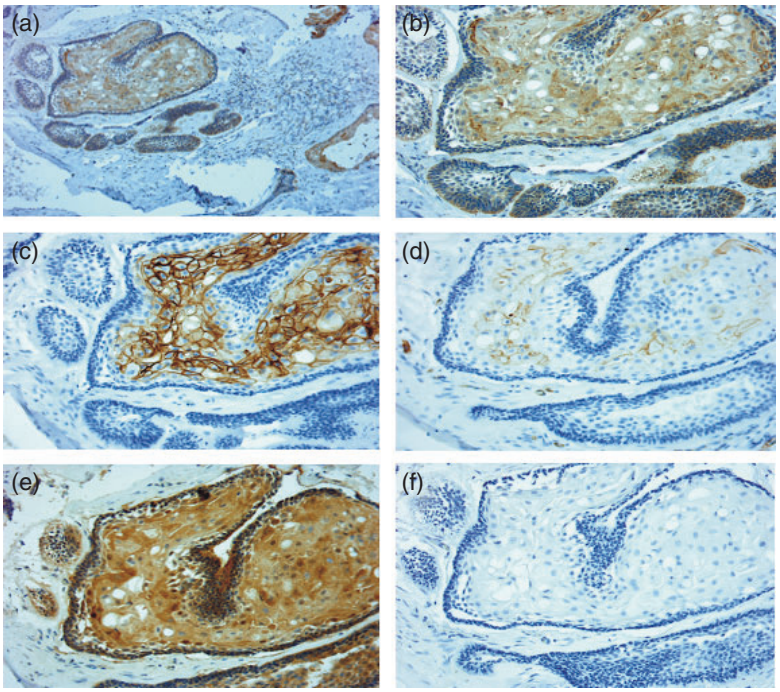

Figure 3 Immunohistochemical reactivity for claudins 1, 4, 5 and 7 and occludin in acanthomatous ameloblastoma. (a and b) Strong immunoreactivity in central squamous cells and moderate immunoreactivity in peripheral cells for claudin 1 (×50 and ×100 respectively). (c) Strong membrane-associated immunoreactivity in central squamous cells with negative immunostaining of peripheral cells for claudin 4 (×100). (d) Weak immunoreactivity in central cells for claudin 5 (×100). (e) Strong central squamous and peripheral cell immunoreactivity for claudin 7 (×100). (f) Negative immunoreactivity for occludin (×100).

Table 1 Immunoreactivity for claudins 1, 4, 5, 7 and occludin in ameloblastomas

	Claudin 1			*Claudin 4*			*Claudin 5*			*Claudin 7*			*Occludin*		
	–	+	+ + +	–	+	+ + +	–	+	+ + +	–	+	+ + +	–	+	+ + +
Follicular ($n = 7$)															
Peripheral cells	1 (14)	4 (**57**)	2 (29)	7 (**100**)	0 (0)	0 (0)	7 (**100**)	0 (0)	0 (0)	0 (0)	2 (29)	5 (**71**)	6 (**86**)	1 (14)	0 (0)
Central cells	1 (14)	3 (**43**)	3 (**43**)	4 (**57**)	2 (29)	1 (14)	5 (**71**)	2 (29)	0 (0)	0 (0)	2 (29)	5 (**71**)	5 (**71**)	2 (29)	0 (0)
Plexiform ($n = 12$)															
Peripheral cells	0 (0)	9 (**75**)	3 (25)	10 (**83**)	2 (17)	0 (0)	11 (**92**)	0 (0)	1 (8)	0 (0)	4 (33)	8 (**67**)	7 (**58**)	5 (42)	0 (0)
Central cells	0 (0)	6 (**50**)	6 (**50**)	0 (0)	10 (**83**)	2 (17)	8 (**67**)	3 (25)	1 (8)	0 (0)	3 (25)	9 (**75**)	6 (**50**)	6 (**50**)	0 (0)
Acanthomatous ($n = 2$)															
Peripheral cells	0 (0)	2 (**100**)	0 (0)	2 (**100**)	0 (0)	0 (0)	2 (**100**)	0 (0)	0 (0)	0 (0)	2 (**100**)	0 (0)	2 (**100**)	0 (0)	0 (0)
Central cells	0 (0)	0 (0)	2 (**100**)	0 (0)	0 (0)	2 (**100**)	0 (0)	2 (**100**)	0 (0)	0 (0)	0 (0)	2 (**100**)	1 (**50**)	1 (**50**)	0 (0)
Mixed ($n = 4$)															
Peripheral cells	0 (0)	4 (**100**)	0 (0)	3 (**75**)	1 (25)	0 (0)	4 (**100**)	0 (0)	0 (0)	0 (0)	4 (**100**)	0 (0)	4 (**100**)	0 (0)	0 (0)
Central cells	0 (0)	2 (**50**)	2 (**50**)	0 (0)	3 (**75**)	1 (25)	1 (25)	3 (**75**)	0 (0)	0 (0)	2 (**50**)	2 (**50**)	0 (0)	3 (**75**)	1 (25)
Ameloblastic carcinoma ($n = 4$)															
Peripheral cells	0 (0)	2 (**50**)	2 (**50**)	2 (**50**)	(25)	1 (25)	2 (**50**)	2 (**50**)	0 (0)	0 (0)	3 (**75**)	1 (25)	4 (**100**)	0 (0)	0 (0)
Central cells	0 (0)	1 (25)	3 (**75**)	0 (0)	2 (**50**)	2 (**50**)	2 (**50**)	2 (**50**)	0 (0)	0 (0)	3 (**75**)	1 (25)	3 (**75**)	1 (25)	0 (0)

Immunohistochemical reactivity: –, negative; +, positive (including both weak and moderate positivity); + + +, strongly positive.
Values in parentheses denote percentage. The highest percentage value in each category is bolded.

the acanthomatous and mixed variants showed mostly moderate immunoreactivities in the peripheral cells whereas the staining in the central areas with squamous differentiation was strong (Fig. 3e and Table 1). In the mixed variant, peripheral cells stained weak in two of four cases, whereas the staining of peripheral cells in ameloblastic carcinoma was weak in three cases. One ameloblastic carcinoma case had, however, a strong staining pattern in the peripheral cells. In the central cells claudin 7 immunoreactivity was predominantly weak (two weak, one moderate), and in two cases there was a strong staining intensity in the mixed form ameloblastomas. The central cells in ameloblastic carcinomas had two cases with weak, one moderate, and one strong staining pattern for claudin 7 (Table 1).

Occludin immunoreactivity was mostly negative in all ameloblastoma types analyzed with some cases showing

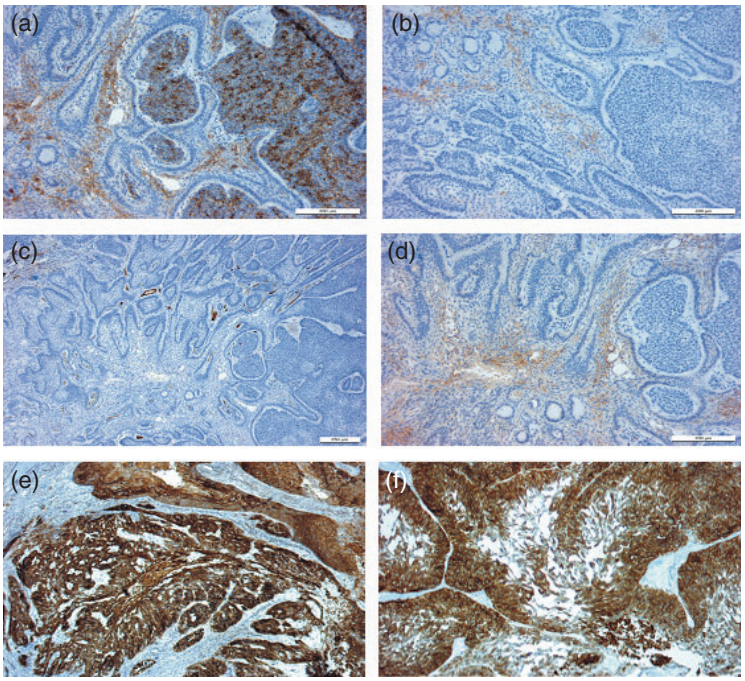

Figure 4 Immunohistochemical reactivity for claudins 1, 4, 5 and 7 in ameloblastic carcinoma. (a) Variable immunoreactivity for claudin 1 (×100). (b) Weak immunoreactivity for claudin 4 (×50). (c) Strong immunoreactivity only in vascular structures for claudin 5. (d) Moderate immunoreactivity in central cells for claudin 7 (×100). (e and f) Strong immunoreactivity for claudin 1 in two malignant ameloblastomas (×100).

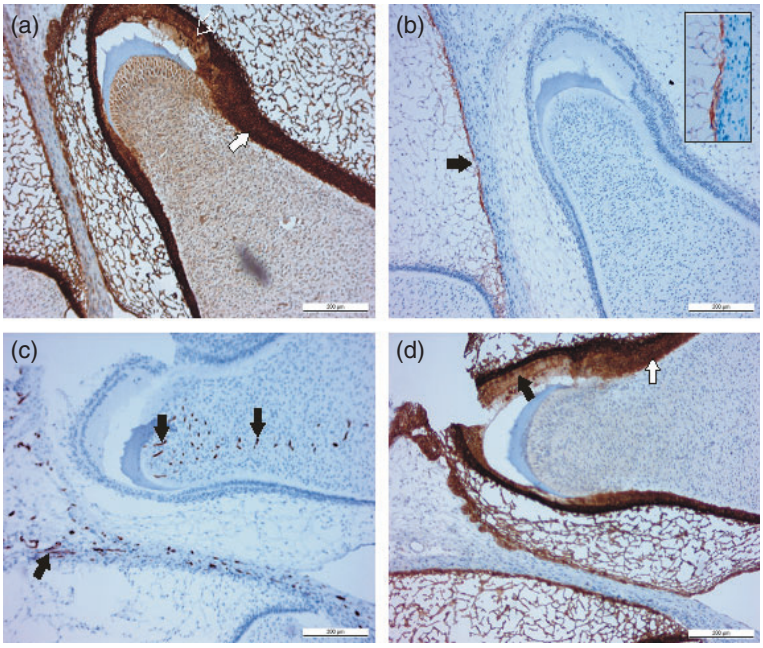

Figure 5 Immunohistochemical reactivity for claudins 1, 4, 5 and 7 in developing human teeth. (a and d) Strong immunoreactivity for enamel organ, ameloblasts, and enamel matrix for claudins 1 and 7, respectively (enamel – dark arrow, ameloblasts – white arrows; ×100). (b) Moderate immunoreactivity of the outer enamel epithelium for claudin 4. Inset is the enlarged version of the area with arrow (×100). (c) Strong immunoreactivity of vascular structures only for claudin 5 (arrows; ×100).

weak immunoreactivity (Figs 1f, 2f and 3f). Moderate staining was seen in mixed variant, which also had the only case with a strong immunoreactivity (Table 1).

Claudins 1, 4, 5 and 7 and occludin in dental germs

The two dental germs used were in the late bell stages. There was intense staining of the inner and outer enamel

epithelium, stratum intermedium, stellate reticulum, ameloblasts, and the newly formed enamel by claudins 1 and 7 (Fig. 5a,d). The ameloblasts demonstrated intense cellular membrane and cytosol staining reaction. The staining reaction was negative for dental papilla and odontoblasts (claudin 7) or relatively weak (claudin 1), and completely negative for newly formed dentine. Staining for claudin 4 was seen only in the outer enamel epithelium and stellate reticulum and was relatively weak (Fig. 5b). Claudin 5 appeared to only preferentially stain the vascular structures present in dental papilla and dental follicle (Fig. 5c). Occludin immunoreactivity was very weak (not shown).

Discussion

Immunohistochemistry was used to study the location of claudins 1, 4, 5 and 7 in benign and malignant ameloblastomas and in human dental germ. All cellular variants of benign solid ameloblastomas analyzed showed strong immunoreactivity for claudins 1 and 7 with the most staining concentrated in the loose polyhedral stellate reticulum-like central cells and particularly in the areas of squamous differentiation. Claudin 4 showed usually moderate immunoreactivity for the central cells and was negative for the peripheral cells except in six cases. Claudin 5 showed weak immunoreactivity for the central cells and was always negative for the peripheral cells of benign ameloblastomas. Immunoreactivity for occludin was negative in most cases and in the few positive cases was weak and found mainly in relation to the central cells.

In previous studies, claudin expression has been studied in a variety of tumors where its expression is mainly found in epithelium-derived and vascular tumors (13). Vascular tumors express mainly claudin 5, but expression of other claudins, such as claudins 2 and 3 was also reported (13). In epithelial tumors, several claudins, such as claudins 1, 2, 3, 4 and 7 are present, but claudin 5 expression has also been reported in several carcinomas (13, 14). In various tumors, claudins may be overexpressed or downregulated. Overexpression of claudin 4 has been shown in breast and ovarian carcinomas (15, 16) while loss of claudin 7 expression has been associated with aggressive behavior in breast carcinoma (17). Using cDNA microarray analysis, it has been shown that claudin 7 expression is downregulated in head and neck squamous cell carcinoma (19). Reduced expression of claudin 7 has also been reported in invasive cervical cancers compared with cervical intraepithelial neoplasia stages 1 and 2 (CIN 1/2; 20), although this is not in agreement with the report of other workers (21). In gastric carcinoma, downregulation of claudins, such as claudin 3, is associated with the diffuse subtype and is also associated with a poor outcome of the patients (18). The immunoreactivity found for claudins in ameloblastoma is in keeping with the reports found in other tumor types showing evident expression of claudins 1, 4 and 7 in these tumors while claudin 5 is less frequent and mainly located in vascular structures. We could not, however, observe any significant differences in their expression between benign or malignant ameloblastic tumors. However, we remain cautious of the fact that only four cases of ameloblastic carcinomas were available for our analysis and that there were staining variations between the cases.

Claudins are membrane-associated proteins which may have relevance in embryonic development and organogenesis by influencing for instance epithelial-mesenchymal transition and may also in this way be involved in tumorigenesis. Because of this it was interesting to compare their expression in ameloblastomas to that of dental germs. The dental germs showed similar pattern of staining for the claudins than ameloblastomas although the immunoreactivity for claudins 1 and 7 in the ameloblasts appeared to be stronger than in the peripheral cells of ameloblastomas. In all ameloblastomas and tooth germs, immunoreactivity was stronger in the central than the peripheral cells. The overexpression of claudins in the central stellate reticulum like cells may indicate their attempt to maintain cell–cell attachments in the loosely arranged islands where formation of microcysts usually occurs. The expression of the claudins 1, 4 and 7 may thus be related to incipient cystic degeneration. Claudin 1 expression is also prominent in the areas of squamous differentiation of ameloblastomas resembling thus the distribution of claudin 1 in squamous epithelium of the skin (22). The strong immunoreactivity of ameloblasts and newly formed enamel matrix for claudins 1 and 7 is also noted. Interestingly, ameloblasts and developing enamel matrix also express anchoring filament laminin-5 (23), basement membrane collagen XVIII (24), and enamel-specific protease enamelysin MMP-20 (24). Enamelysin cleaves both collagen XVIII (23) and laminin-5 gamma2 chain (25), and processed form of laminin-5 gamma2 chain induces epithelial cell migration (25). It is possible that processed form of laminin-5 also influences ameloblasts' migration and production of enamel and shaping of enamel prisms during tooth development. Claudin 1 has recently been shown to induce the expression of MMP-14 and -2 leading to enhanced cleavage of laminin-5 gamma2 chain and upregulation of epithelial cancer cell invasion (26). The physiologic roles of claudins in the enamel, ameloblasts, and ameloblastomas still remain to be proven but may well be, at least partially, mediated through the activation of various MMPs leading to controlled enamel matrix formation or growth of odontogenic tumors.

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