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Original

Heterogeneous and abnormal localization of desmosomal proteins in oral intraepithelial neoplasms

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Abstract: To study the relationship between the biological and morphological characteristics of oral intraepithelial neoplasms (OINs), we examined the localization of desmosome-related proteins. Twenty-seven cases of OIN3 were tentatively classified as basaloid (14 cases) or differentiated (13 cases), and the latter were further subdivided into verrucous (five cases) and acanthotic (eight cases) subtypes. All samples were stained using antibodies against desmoglein 1 (DSG1), desmocollin 3 (DSC3), junction plakoglobin (JUP) and serine peptidase inhibitor Kazal type 5 (SPINK5) domain. All variants of OIN3 showed significantly high rates of positivity for DSG1 in the basal layer (basaloid 57%; differentiated 85%), DSC3 in the surface layer (basaloid 93%; differentiated 77%) and JUP in the basal and parabasal layers (basaloid 93%; differentiated 62%). Interestingly, even the basaloid type showed areas of alternating DSG1 positivity and negativity, reflecting keratinocyte maturation. Therefore, most cases of OIN appear to have the characteristics of well differentiated squamous epithelium. (J Oral Sci 56, 209-214, 2014)

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Introduction

Oral intraepithelial neoplasms (OINs), including carcinoma *in situ*, show variable histological features (1). Since the 1960s, it has been accepted that carcinoma *in situ* can be narrowly defined as complete disordering through all layers of the epithelium. The maturation of surface layers has not been taken into account in the diagnosis of oral carcinoma *in situ*. In fact, the lesion demonstrates varied morphological changes with or without keratinization (2). However, the World Health Organization (WHO) diagnostic criteria for carcinoma *in situ* do not consider histological variations (3).

Objective diagnosis of borderline malignancies may be challenging, involving gene profiling or screening for the presence of abnormal proteins. Cytokeratin is a helpful diagnostic marker for evaluation of cellular differentiation in tumors (4), and cytokeratins 13 and 19 in particular are useful for diagnosis or biological characterization of oral precancerous lesions or carcinomas *in situ* in squamous epithelium (5,6). However, the distribution of cytokeratin differs among the various types of oral mucosa, such as those overlying the tongue and the gingiva, and thus shows a degree of heterogeneity.

The desmosome is composed of various proteins,

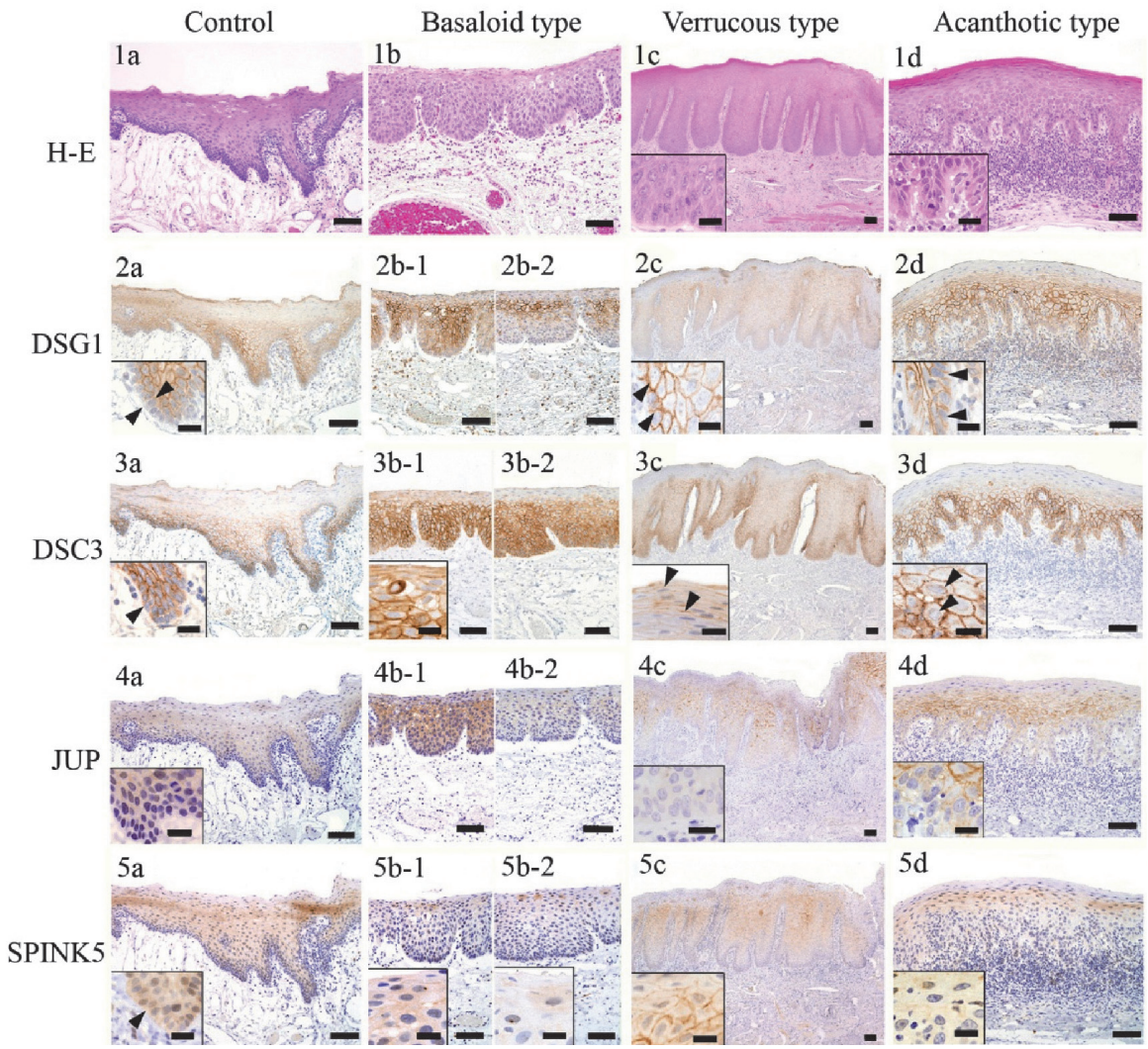

Fig. 1

1. H-E staining shows variable features of control specimens (a), and basaloid-type (b) and differentiated-type (c: verrucous and d: acanthotic) OIN with atypical cells (insets).
2. Desmoglein 1 (DSG1) is negative in basal cells (inset, arrowheads) of a control specimen (a), shows variable immunoreactivity in basaloid-type OIN (b), and is positive in basal cells in differentiated-type OIN (c: verrucous and d: acanthotic) (insets, arrowheads).
3. Desmocollin 3 (DSC3) is positive in basal cells (inset, arrowhead) of a control specimen (a), and shows constant immunopositive reaction in basaloid-type (b) and differentiated-type OIN (c: verrucous and d: acanthotic). Surface layers are also positive (inset, arrow heads) (c) with perinuclear positive reaction (d) (inset, arrowheads).
4. Plakoglobin (JUP) is positive in a control specimen (a), shows variable immunoreactivity in basaloid-type OIN (b), and negative reaction of lower layers in differentiated-type OIN (c: verrucous and d: acanthotic) (insets).
5. Serine peptidase inhibitor Kazal type 5 (SPINK5) is positive in all layers of a control specimen (inset, arrowhead) but shows varied positivity (insets) in basaloid (b) and differentiated-type OIN (c: verrucous and d: acanthotic).

Bars indicate 100 μm and 20 μm in low-magnification photos and high-magnification insets, respectively.

including desmoglein, desmocollin, desmoplakin, plakofilin, and plakoglobin, which have cell-cell anchoring functions. The distribution of desmosomal proteins differs according to the function and type of the epithelium, and also in some diseases (7). Abnormal changes in desmosomal proteins occur not only in various head and neck squamous cell carcinomas (8-11) but also in borderline malignancies of the cervix (12) and oral mucosa (13,14). However, only a limited number of studies have investigated alterations of desmosomal proteins in oral intraepithelial neoplasms.

Therefore, the purpose of the present study was to

Table 1 The panel of primary antibodies

Antibody	Source	Clone	Treatment	Dilution
desmoglein 1	PROGEN, Heidelberg, Germany	Mab Dsg1-P23	HIE, pH6	Ready to use
desmocollin 3	PROGEN, Heidelberg, Germany	Mab Dsc3-U114	HIE, pH6	1 : 10
plakoglobin (γ-catenin)	SANTA CRUZ, Santa Cruz, CA, USA	Mab SC-8415	No pretreat	1 : 50
serine peptidase inhibitor, Kazal type 5	Atlas Antibodies, Stockholm, Sweden	Rab poly	HIE, pH6	1 : 250

Mab: mouse monoclonal antibody, Rab poly: rabbit polyclinal antibody, HIE: heat induced epitope retrieval

examine the localization of desmosomal proteins in oral intraepithelial neoplasms and to clarify the relationship between their localization and histological variation.

Materials and Methods

Case selection

The specimens were selected from archived cases diagnosed at the Laboratory of Surgical Pathology, Matsumoto Dental University Hospital. As a control group, we selected 10 specimens of oral mucosa excised from the gingiva (five cases) and the lateral surface of the tongue (five cases) that did not show significant histological changes, with or without slight inflammation (Fig. 1-1a). The studied specimens were 27 cases of oral borderline malignancies that were consistent with oral intraepithelial neoplasms 3 (OIN3) (15), including severe dysplasia and carcinoma *in situ*, in the WHO classification (3). Twenty-two, four, and one of these specimens had been excised from the tongue, gingiva and palate, respectively.

OIN3 cases were tentatively classified into the basaloid (14 cases) and differentiated (13 cases) types, and the latter being further subdivided into the verrucous (five cases) and acanthotic (eight cases) types. Briefly, the basaloid type showed replacement by atypical basaloid cells similar to cervical intraepithelial neoplasia (Fig. 1-1b). Among the differentiated types, the verrucous subtype had rounded rete processes and showed gradual differentiation toward the surface (Fig. 1-1c), and the acanthotic subtype often showed distinctive keratinization on the surface with steeply serrated rete processes (Fig. 1-1d).

Tissue preparation and immunohistochemistry

All specimens were fixed in 10% buffered formalin, and routinely processed into 3-μm-thick paraffin sections for histological examination. High-temperature unmasking was performed by autoclaving at 121°C for 15 min in 0.01 M sodium citrate buffer solution (pH 6.0), and some sections were digested with proteinase K (Dako, Glostrup, Denmark). As shown in Table 1, primary antibodies against desmoglein 1 (DSG1), desmocollin 3 (DSC3), junction plakoglobin (γ-catenin; JUP) and serine peptidase inhibitor Kazal type 5 (SPINK5) domain, and Nichirei MAX-PO Multi (Nichirei, Tokyo, Japan) as a secondary antibody were incubated at room temperature using HISTOSTAINER (Nichirei) for 60 min and 30 min, respectively. After visualization with 3-3'-diaminobenzidine tetrahydrochloride (Dako), sections were counterstained with hematoxylin. Negative control slides were processed without the primary antibodies.

Evaluation of immunohistochemical staining

Cells showing weak, medium, and strong membranous immunoreactions were equally evaluated as positive, while lack of any membranous reaction was considered to be negative. Finally, layers in which 50% of cells or more were immunopositive were judged as 'positive'.

Statistical analysis

The positivity rate in each layer was analyzed using Fisher's exact test and corrected by Holm's method. Differences at $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed with a modified version of R commander (EZR version 1.6-3, Saitama Medical Center, Jichi Medical University, Saitama, Japan) (16), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13).

Ethics

This study was approved by the Ethics Committee of Matsumoto Dental University (#0127, 2010) and conducted according to the principles of the Helsinki Declaration (sixth version, 2008).

Results

Localization of desmosome-related proteins

In all of the controls, parabasal to surface layers were immunopositive for DSG1, but basal cells were mostly negative (Fig. 1-2a). Among the basaloid and differentiated types, basal cells were DSG1-positive in 57% ($P < 0.05$) and 77% ($P < 0.001$) of cases, the difference being significant (Figs. 1-2b and 2c, Table 2). Proliferated basa-

Table 2 DSG1, DSC3, JUP, and SPINK5 Localizations in OIN3

		Control $n = 10$	Basaloid $n = 14$	Differentiated $n = 13$
DSG1	surface	10	14	11 (85)
	spinous	10	14	13
	parabasal	10	10 (71)	12 (92)
	basal	0	8 (57)*	10 (77)***
DSC3	surface	0	13 (93)***	10 (77)***
	spinous	10	14	13
	parabasal	10	10 (71)	13
	basal	10	14	12 (92.3)
JUP	surface	10	12 (86)	13
	spinous	10	14	10 (77)
	parabasal	10	13 (93)	8 (62)*
	basal	10	13 (93)	8 (62)*
SPINK5	surface	10	9 (64)	13
	spinous	10	6 (43)***	12 (92)
	parabasal	10	3 (21)***	6 (46)**
	basal	10	0***	0***

The values are given as *n* (%) of positive cases.
* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, significant difference compared with the control by Fisher's exact test with Holm's correction.

loid cells showed varying DSG1 reactivity in the lower half of the epithelium, with intermingling of strongly positive and negative areas (Fig. 1-2b).

All control specimens were positive for DSC3 in the lower three layers but negative in the surface layer (Fig. 1-3a). In OIN3, DSC3 was most strongly expressed basally, and its staining intensity declined gradually toward the upper layers, while the surface layer showed weak positivity (Figs. 1-3b, 3c, and 3d). Some cells showed abnormal cytoplasmic accumulation or perinuclear localization of DSC3 (Figs. 1-3b and 3d, insets). These features were also evident in sections stained for DSG1 and JUP (data not shown). Surface DSC3 positivity in OIN3 was significantly higher than that of the control ($P < 0.001$) (Table 2).

The basal and surface layers in control specimens were weakly positive for JUP, but a positive reaction was observed in all layers (Fig. 1-4a). The basaloid type showed a mixture of positive and negative areas, similar to the features revealed by DSG1 immunostaining, but most cases were JUP-positive (Fig. 1-4b, Table 2). On the other hand, in the differentiated type, the basal and parabasal layers were sometimes negative for JUP, and consequently the JUP positivity rate was decreased to 62% ($P < 0.05$) (Fig. 1-4b, Table 2).

SPINK5 immunostaining revealed various degrees of positivity in the control specimens. The upper spinous layer showed the strongest immunoreactivity among the various layers (Fig. 1-5a). In the basaloid type, proliferated cells with basaloid features were mostly negative for SPINK5 (Fig. 1-5b), and in the differentiated type the lower layers also lacked SPINK5 positivity (Figs. 1-5c and 5d). The degree of SPINK5 positivity in OIN3 was significantly different from that of the controls, especially in the basal layer ($P < 0.001$) (Table 2).

Discussion

DSG1 and DSC3 are major Ca^{2+}-dependent transmembrane glycoprotein molecules that anchor adjacent epithelial cells to one another. The molecular distribution of desmosomes varies among different cell types (7): DSG has four isotypes and DSC has three. Simple epithelia express only DSG2 and DSC2, whereas stratified squamous epithelium express all isotypes of DSG and DSC in different patterns (7). Narayana et al. observed abnormal cytoplasmic staining of DSG1 similar to that shown in the present study, but found no significant decrease of DSG1 reactivity in OINs (14). Lo Muzio and coworkers also reported loss of exclusive membranous expression and cytoplasmic delocalization of γ-catenin (i.e. JUP) in OINs (13). Our data showed that the localizations of DSG1 and DSC3 in OIN3 were different from those in control specimens, suggesting that desmosomal cadherins could be potential markers of abnormal differentiation in oral epithelia.

DSG1 is commonly detected at the interface between basal and suprabasal keratinocytes. This cadherin is first expressed in the epidermis as keratinocytes transit from the basal layer (17), whereas DSC3, but not DSC1, is expressed in the desmosomes of basal cells as well as the suprabasal cell layers of stratified epithelia such as those of the vagina, tongue and esophagus (18). Immunofluorescence staining has shown that the intensity of DSC3 expression fades gradually in the suprabasal layers and completely disappears in the upper limit of spinous layer, even though the mRNA is expressed throughout the full thickness of the epithelium (19). These staining patterns were basically the same as those revealed in our present control specimens.

The DSG1(-) to DSG1(+) transition shown by basaloid cells might reflect a switch to the characteristics of parabasal or spinous keratinocytes, because DSG1 has the ability to promote keratinocyte differentiation (17). Considering this change in DSG1 expression, the first layer of the differentiated type showing basal cell alignment is positive for DSG1, which is not the true nature of basal cells. This abnormality can be observed by cytokeratin immunostaining, and the first layer of the

differentiated type lacks cytokeratin 19 (5). Therefore, differentiated OIN3 is considered to be a form of intraepithelial neoplasia showing full-thickness replacement by differentiated keratinocytes.

The mixture of positive and negative areas of DSG1 and JUP in the basaloid type was also noteworthy. Atypical proliferating basaloid cells replaced more than two thirds of the lower epithelium, which may have reflected a status more advanced than the so-called "two-phase appearance" that has been reported to characterize oral moderate dysplasia (5). Nearly 50% of the basaloid-type specimens showed complete loss of DSG1 immunostaining in the lower two thirds of the epithelium, composed of proliferating basaloid cells. As these cases were both morphologically and immunophenotypically similar to basal cells, this type of OIN3 is considered to be the oral counterpart of carcinoma *in situ* of the uterine cervix (12). However, 57% of the basaloid-type specimens showed prominent proliferation of DSG1(+)/DSC3(+)/JUP(+) basaloid cells. Consequently, most cases of OIN3 with basaloid features may have a keratinocytic rather than a basaloid nature.

As mentioned above, DSG1 abnormality was found in all types of OIN3. Therefore, we examined the presence of SPINK5, which is strongly expressed by differentiated keratinocytes in normal skin, and controls its target proteases such as the kallikrein-related peptidases (KLKs). Immunohistochemically, this inhibitor can be detected intracellularly and extracellularly in epidermal layers deeper than those where KLKs are expressed (20). Although DSG1 was aberrantly localized in the basal layer of OIN3, SPINK5 was negative in basal cells. This suggests that overexpression of DSG1 is not attributable to SPINK5 abnormality. If DSG1 overexpression were caused by loss of serine protease, then atypical cells would also overexpress SPINK5. As down-regulation of SPINK5 has been reported previously in head and neck squamous cell carcinomas (21,22), this phenomenon might be one of the biological changes characterizing OIN3. However, its significance remains unclear.

With regard to expression of DSC3, the surface layer in all OIN3 cases showed significantly high rates of positivity. Wang et al. (9) suggested that the level of DSC3 expression in oral squamous cell carcinoma might be related to cell proliferation. Furthermore, in basaloid-type OIN3, DSC3 was consistently localized in all cell layers. Transgenic mice with suprabasal overexpression of DSC3 driven by the keratin 1 promoter have been reported to show acanthosis and increased proliferation of basal cells. Taken together, these results suggest that DSC3 may contribute to the proliferation and differentiation of keratinocytes in OINs.

It is unclear why JUP expression was reduced in the basal and parabasal layers of differentiated-type OIN. JUP is a member of the armadillo family that participates in not only adhesion but also signaling (23). Furthermore, JUP deficiency protects keratinocytes against apoptosis (24). Accordingly, low JUP expression in basal cells may correlate with a well differentiated and verrucous morphology. However, because the present sample size for this subtype was too small for precise analysis, examination of more cases will be necessary.

Oral squamous cell carcinomas tend to be well differentiated, and as mentioned above, OINs also show a well differentiated nature. On this basis, the WHO criterion for carcinoma *in situ*, i.e. "full-thickness architectural abnormally" appears to be insufficient (1), and for precise diagnosis, variations in differentiation such as the presence of verrucous or acanthotic features should be borne in mind.

In conclusion, alterations of desmosomal protein localization appear to reflect the heterogeneous phenotypic and morphologic nature of OIN3, and neoplastic proliferation of keratinocytes.

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