

Immunohistochemical Analysis of Oral Dysplasia: Diagnostic Assessment by Fascin and Podoplanin Expression

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The aim of this study was to investigate fascin and podoplanin expression in oral dysplasia and carcinoma *in situ* (CIS) immunohistochemically, and to evaluate their relationship to histopathological diagnosis based on architectural and cytological features. Fascin and podoplanin expression patterns were analyzed immunohistologically in 26 specimens of oral lesions, including benign disease (hyperplasia, papilloma, and others), intraepithelial neoplasia/borderline disease (dysplasia), and malignant disease (CIS, invasive squamous cell carcinoma). Fascin expression was scored into four original categories, and podoplanin expression was scored into five previously established categories. The relationship between the immunohistochemically determined scores of fascin and podoplanin expression and the architectural and cytological features in the hematoxylin-eosin-stained slides was analyzed statistically. The immunostaining scores for fascin and podoplanin were significantly higher in dysplasia and CIS than in benign disease ($p=0.0011$, $p=0.00036$), and they were significantly higher in dysplasia than in benign disease ($p=0.0087$, $p=0.0032$). In all cases of invasive SCC, fascin was expressed mainly in the cytoplasm of the tumor cells and fascin expression extended from the destruction of the basal layer of the epithelium to the upper layer of the epithelium and podoplanin was expressed in the cytoplasm and membrane of the tumor cells. This was the first report of up-regulation of fascin in oral dysplasia. Our results suggest that it would be helpful for improving the diagnostic accuracy of oral dysplasia and CIS to assess the expression of fascin and podoplanin immunohistochemically.

Key words: fascin, podoplanin, oral dysplasia, CIS, immunohistochemistry

I. Introduction

In the WHO classification, epithelial precursor and related lesions are histopathologically classified into squamous cell hyperplasia and mild, moderate, and severe dysplasia and carcinoma *in situ* (CIS) by the assessment of the presence and severity of epithelial dysplasia based on

architectural and cytological features [5]. Oral dysplasia typically progresses through a sequence of histopathological lesions, including mild, moderate to severe dysplasia, CIS, and finally invasive cancer. Oral leukoplakia (OL) is 'a white patch' diagnosed by clinical finding and gross pathology, that includes hyperplasia, dysplasia, CIS, invasive squamous cell carcinoma (SCC), and other diseases histopathologically. The progress rates into invasive SCC of OL diagnosed with hyperplasia or dysplasia histopathologically have been reported to range from 17% in a series of patients followed-up for 8 years [29] to 31% in a series followed-up for 7 years [17]. Because the 5-year survival rate of oral squamous cell carcinoma (OSCC) is around

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50%, and the tumor-node-metastasis (TNM) stage at presentation significantly affects 5-year survival, early diagnosis and treatment are crucial for reducing mortality from this disease [31]. Therefore, we consider that appropriate diagnosis and management of epithelial precursor lesions may allow us to manage appropriately the intra-epithelial neoplasia/borderline disease or to provide early treatment of cancer and thereby help to decrease the number of deaths from OSCC.

However, it has been reported that histopathological assessment of the presence and severity of epithelial dysplasia based on architectural and cytological features is inconsistent, with substantial levels of inter- and intra-observer variation [1, 13]. Further, it is often difficult to distinguish between dysplasia and other benign lesions, because oral benign lesions other than dysplasia also frequently show architectural disturbance and cytological atypia. Therefore, in addition to assessing epithelial dysplasia morphologically, objective biomarkers that do not require the ability to recognize morphologic changes would be helpful for a more precise histopathological diagnosis of dysplasia. In this context, we have become interested in fascin and podoplanin as potentially key factors for the diagnosis of dysplasia.

Fascin, which is an actin-bundling protein, plays a role in cell transformation and in increasing cell motility [2]. The expression of fascin protein is often up-regulated in several types of human neoplasm, including oral [4, 18], breast [7, 35], pancreatic [19], colonic [12], and lung tumors [24], and the overexpression of fascin has been implicated in tumor progression [11]. However, there have been no studies on fascin expression in oral dysplasia.

Podoplanin, which belongs to the family of type-1 transmembrane sialomucin-like glycoproteins, is specifically expressed in the lymphatic vascular endothelium [3, 23, 25]. It is also expressed in tumor cells of various types of cancer, such as SCC in several organs [16, 26, 27, 36], germ cell tumors [27], vascular tumors [8], malignant mesothelioma [15], and tumors of the central nervous system [21], and has been shown to play a role in tumor invasion and metastasis through its ability to remodel actin in the cytoskeleton of tumor cells [32]. In addition, it was reported that podoplanin was overexpressed in oral dysplasia and lichen planus, which the World Health Organization (WHO) classifies as being potentially malignant disorders [14, 28]. And recently, the role of podoplanin in early oral tumorigenesis has also begun to attract attention.

The aim of this study was to search for objective

Table 1. Patient's histopathological data

Case No.	Age, years	Sex	location	Histopathology by HE	Immunohistochemical score	
					Fascin	Podoplanin
1	71	M	Tongue	Hyperplasia	0	0
2	84	M	Tongue	Hyperplasia	0	0
3	36	M	Tongue	Fibroepithelial polyp	1	0
4	53	M	Tongue	Fibroepithelial polyp	1	0
5	64	M	Tongue	Fibroma	0	0
6	74	F	Tongue	Fibroma	1	0
7	71	M	Tongue	Papilloma	1	1
8	53	F	Tongue	Papilloma	2	0
9	64	M	Tongue	Papilloma	3	1
10	37	F	Tongue	Mild dysplasia	3	1
11	67	M	Lower alveolus	Mild dysplasia	3	3
12	72	M	Tongue	Mild dysplasia	3	4
13	37	M	Tongue	Moderate dysplasia	3	1
14	44	M	Tongue	Severe dysplasia	2	4
15	44	F	Tongue	Severe dysplasia	3	4
16	74	M	Tongue	Severe dysplasia	3	4
17	66	M	Tongue	Carcinoma <i>in situ</i>	3	4
18	72	F	Tongue	Carcinoma <i>in situ</i>	3	4
19	62	F	Tongue	Carcinoma <i>in situ</i>	3	4
20	63	F	Tongue	Carcinoma <i>in situ</i>	3	4
21	58	F	Hard palate	Invasive SCC	‡	‡
22	68	F	Upper alveolus	Invasive SCC	‡	‡
23	38	M	Tongue	Invasive SCC	‡	‡
24	64	M	Tongue	Invasive SCC	‡	‡
25	66	M	Tongue	Invasive SCC	‡	‡
26	81	M	Tongue	Invasive SCC	‡	‡

M/F, male/female; SCC, squamous cell carcinoma; ‡ are impossible to score.

biomarkers to improve the diagnostic accuracy for epithelial dysplasia. To this end, we examined the expression patterns of fascin and podoplanin immunohistochemically in dysplasia and CIS of oral lesions, and evaluated their relationship to the histopathological diagnosis based on architectural and cytological features.

II. Materials and Methods

Patients and tissue specimens

Tissue specimens of 26 patients were collected from Saitama Medical University Hospital and Saitama Medical University International Medical Center from April 2005 to August 2010 (Table 1). Surgically removed specimens or biopsy specimens were included in the current study. Patients were diagnosed with dysplasia and CIS histopathologically by the 2005 WHO classification. Patients with benign disease (2 cases of hyperplasia, 2 cases of fibroma, 2 cases of fibroepithelial polyp, 3 cases of papilloma) or malignant carcinoma (6 cases of invasive SCC) were selected as comparison subjects.

The average age of the patients was 60.9 (range 36–84) years. Seventeen of the patients were male and nine were female. Tissue specimens included 23 tongue lesions, 2 gingival lesions, and 1 palate lesion. This study was approved by an ethics review board and informed consent was obtained from patients.

Tissue processing

Tissue sections (4 μm thick) from formalin-fixed, paraffin-embedded tissue blocks of surgical specimens or biopsy specimens were mounted on positively charged glass slides.

Immunohistochemical procedure

Immunohistochemistry was performed using the avidin-biotin peroxidase complex technique. In brief, slides were deparaffinized through a series of xylene baths and rehydrated with graded concentrations of alcohol. The slides were then steamed with 10 mmol/L citrate buffer (pH 6.0; DAKO Cytomation, Carpinteria, CA) for 20 min, immersed in methanol containing 3% hydrogen peroxide for 10 min, and incubated in 10% horse serum for 30 min at room temperature. The slides were incubated with specific primary antibodies raised against fascin (mouse monoclonal antibody, clone 55K-2; DAKO) at a 1:100 dilution and podoplanin (mouse monoclonal antibody, clone NZ-1; AngioBio, Del Mar, CA) at a 1:200 dilution at 37°C for 32 min. Immunostaining with an *i*VIEW DAB Detection Kit (Ventana Medical Systems Inc., AZ) and BenchMark XT (Ventana Medical Systems) was performed. Adjacent normal-appearing endothelial cells of blood vessels were used as positive controls for fascin [6], and adjacent normal-appearing lymphatic endothelial cells within the sections served as positive controls for podoplanin [3, 23, 25].

Immunohistochemical evaluation

Cells showing any cytoplasmic immunoreactivity were considered positive for fascin expression. To score the fascin expression, we used a newly developed system with four original categories based on the level of epithelial staining (Fig. 1, upper panel). The expression was scored as 0 if no expression was observed in any part of the epithelium; 1 if expression was restricted to one-third of the epithelium beginning in the basal layer; 2 if expression was observed in two-thirds of the epithelium beginning in the basal layer; and 3 if expression was observed in all areas of the

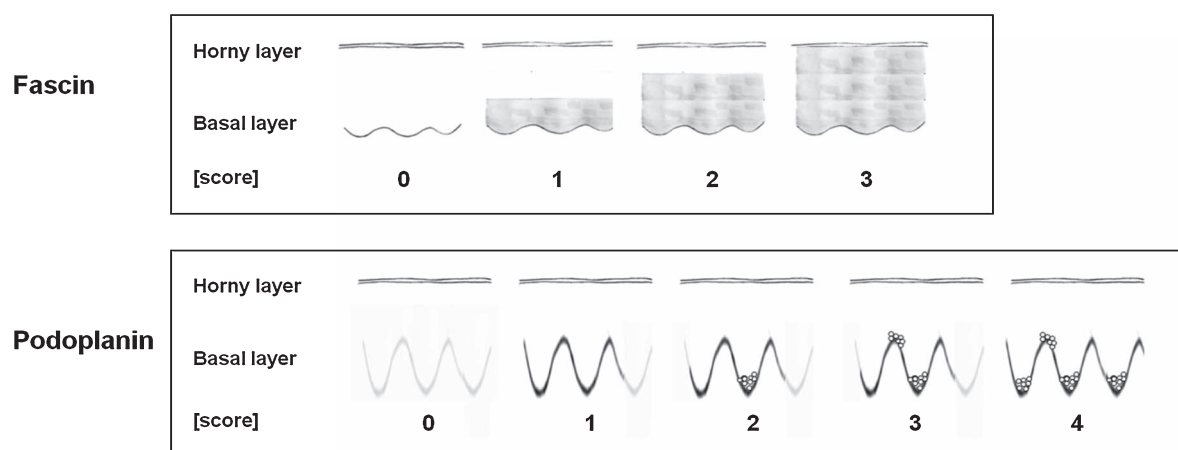


Fig. 1. Schema of the expression patterns with scores. The patterns of expression scores for fascin and podoplanin. The upper panel shows representative expression profiles of fascin corresponding to scores 0 to 3. Score 0: no expression observed in any part of the epithelium. Score 1: expression restricted to one-third of the epithelium beginning in the basal layer. Score 2: expression observed in two-thirds of the epithelium beginning in the basal layer. Score 3: expression observed in all areas of the epithelium. The lower panel shows representative expression profiles of podoplanin corresponding to scores 0 to 4. Score 0: no expression observed in any part of the epithelium. Score 1: expression restricted to the basal layer of the epithelium. Score 2: expression observed in the basal and suprabasal layers in one area. Score 3: suprabasal layer expression is observed in two or three areas. Score 4: suprabasal layer expression is observed in more than three areas.

epithelium.

Cells showing any membrane immunoreactivity were considered positive for podoplanin expression. Podoplanin expression was scored into five categories based on the staining level of the epithelium, as reported by Kawaguchi *et al.* (Fig. 1, lower panel) [6]. The expression was scored as 0 if no expression was observed in any parts of the epithelium; 1 if expression was restricted to the basal layer of the epithelium; 2 if expression was observed in the basal and suprabasal layers in one area in a slice preparation; 3 if suprabasal layer expression was observed in two or three areas in a slice preparation; and 4 if suprabasal layer expression was observed in more than three areas in a slice preparation.

The whole sections were stained with the classic hematoxylin-eosin (HE) staining technique and diagnosed histopathologically by experienced pathologists. When examining the histopathology of the sections, classifications CIS, OSCC, or mild, moderate, or severe dysplasia were histopathologically graded according to the latest WHO guidelines [5].

Statistical analysis

The relationship between the immunohistochemically determined scores of fascin and podoplanin expression and the architectural and cytological features in HE-stained slides was analyzed using the Wilcoxon rank sum test. P values <0.05 were considered to be statistically significant.

III. Results

Correlation between fascin expression and architectural and cytological features

Fascin expression in interstitial tissue was consistently detected in endothelial cells of the vessels (arrows in Fig. 2F, HE staining: Fig. 2A). Fascin expression in the normal squamous epithelium was extremely low in basal cells (not shown). Because fascin was mainly expressed in the

cytoplasm in oral lesions (Fig. 2K–M), we observed wide fascin expression throughout the basal layer of the epithelium (Fig. 2G–I, HE staining: Fig. 2B–D). In Figure 2G, the fascin expression is partially restricted to under one-third of the epithelium beginning in the basal layer. In Figure 2I, we show fascin expression in all areas of the epithelium.

Among the samples from subjects with benign disease (hyperplasia, fibroepithelial polyp, fibroma, or papilloma), 3 out of 9 had no detectable fascin expression, and 4 out of 9 showed fascin expression only in one-third of the epithelium beginning in the basal layer (scored as 1) (Table 1). Fascin expression in two-thirds of the epithelium beginning in the basal layer (scored as 2) and fascin expression in all areas of the epithelium (scored as 3) were each seen in 1 out of the 9 cases.

In the cases with dysplasia, the fascin-expression scores were a relatively high 2 or 3: 1 out of the 7 cases showed fascin expression in two-thirds of the epithelium beginning in the basal layer (scored as 2), and 6 out of 7 showed fascin expression in all areas of the epithelium (scored as 3). In all cases (4/4) of CIS, fascin was expressed in all area of the epithelium (scored as 3).

The immunostaining scores for fascin were significantly higher in dysplasia and CIS than in the benign diseases (hyperplasia, fibroepithelial polyp, fibroma, or papilloma) ($p=0.0011$). Further, the immunostaining scores for fascin were significantly higher in dysplasia than in the cases of benign disease (hyperplasia, fibroepithelial polyp, fibroma, or papilloma) ($p=0.0087$). The immunostaining scores for fascin were not statistically different between dysplasia and CIS ($p=0.45$).

Correlation between the podoplanin expression and architectural and cytological features

As expected, podoplanin was highly expressed in the endothelial cells of lymphatic vessels (arrows in Fig. 3G, HE staining: Fig. 3A), but was not detectable in the endothelial cells of blood vessels. Podoplanin expression

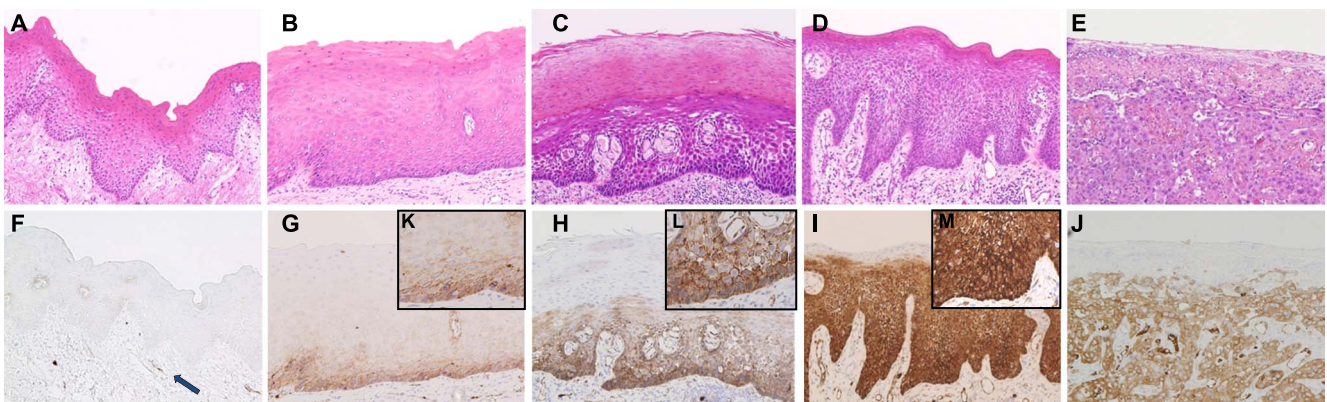


Fig. 2. Expression patterns of fascin. A–E: Hematoxylin and eosin staining. F–L: Expression patterns of fascin. The blue arrows indicate blood vessels. The square indicates magnified images. Magnification $\times 100$ (A–J), $\times 400$ (K–M). A, F: score 0, hyperplasia; B, G, K: score 1, mild dysplasia; C, H, L: score 2, severe dysplasia; D, I, M: score 3, carcinoma *in situ*; E, J: invasive SCC.

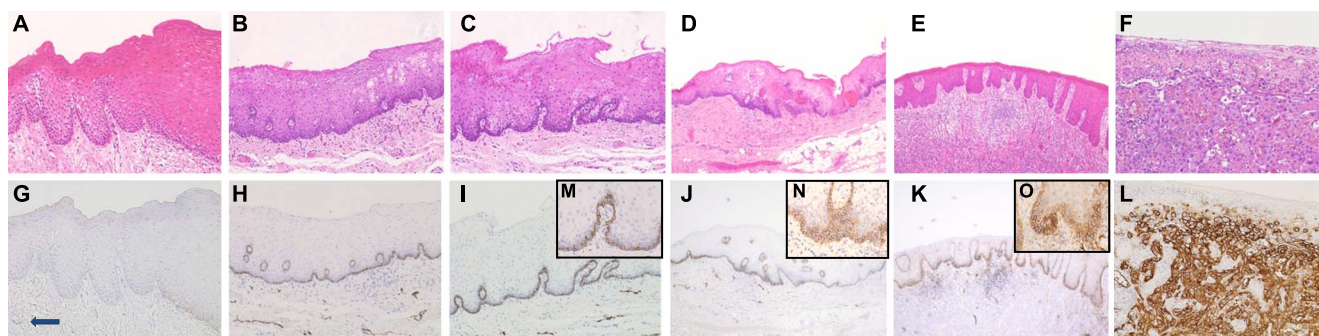


Fig. 3. Expression patterns of podoplanin. A–F: Hematoxylin and eosin staining. G–O: Expression patterns of podoplanin. The arrows indicate lymphatic endothelial cells. The square indicates magnified images with positive podoplanin expression. Magnification $\times 100$ (A–C, F, G–I, L), $\times 40$ (D, E, J, K), $\times 400$ (M–O). A, G: score 0, hyperplasia; B, H: score 1, mild dysplasia; C, I, M: score 2, mild dysplasia; D, J, N: score 3, mild dysplasia; E, K, O: score 4, carcinoma *in situ*; F, L: invasive SCC.

was not detectable in the normal squamous epithelium (not shown). In oral lesions, podoplanin expression was highly variable in the epithelium (Fig. 3H–K, HE staining: Fig. 3B–E). Podoplanin was expressed mainly in the membrane region of the basal layer (Fig. 3M–O). In some cases, podoplanin was highly expressed on the cell membrane predominantly at the basal layer (Fig. 3H); in other cases the expression extended to the suprabasal layer (Fig. 3I–K).

In the benign diseases (hyperplasia, fibroepithelial polyp, fibroma, papilloma), podoplanin expression was low, with scores of 0 or 1: 7 of 9 cases showed no detectable podoplanin expression in the epithelium (scored as 0), and 2 out of 9 showed podoplanin expression only in certain basal layer cells (scored as 1) (Table 1).

In dysplasia, podoplanin expression was variable in the epithelium: 2 out of 7 cases showed podoplanin expression only in certain basal layer cells (scored as 1), 1 out of 7 showed podoplanin expression in the suprabasal layer in two or three areas (scored as 3), and 4 out of 7 showed podoplanin expression in the suprabasal layer in more than three areas (scored as 4). In all cases (4/4) of CIS, podoplanin expression was given the highest score of 4: present in the suprabasal layer in more than three areas.

The immunostaining scores for podoplanin were significantly higher in dysplasia and CIS than in the benign disease (hyperplasia, fibroepithelial polyp, fibroma, or papilloma) ($p=0.00036$). Further, the immunostaining scores for podoplanin were significantly higher in dysplasia than in the benign disease (hyperplasia, fibroepithelial polyp, fibroma, or papilloma) ($p=0.0032$). The immunostaining scores for podoplanin were not statistically different between dysplasia and CIS ($p=0.15$).

Fascin and podoplanin expression in invasive OSCC

Because the tumor cells invaded into the lamina propria, destroying the basal layer of the epithelium, we were unable to score the fascin and podoplanin expression in invasive SCC (Table 1). But in all cases (6/6) of invasive SCC, fascin was expressed mainly in the cytoplasm of the tumor cells and fascin expression extended from the destruction of the

basal layer of the epithelium to the upper layer of the epithelium (Fig. 2J, HE staining: Fig. 2E). Further, in all cases (6/6) of invasive SCC, podoplanin was expressed in the cytoplasm and membrane of the tumor cells (Fig. 3L, HE staining: Fig. 3F).

IV. Discussion

Fascin has a major function in forming the parallel actin bundles that support lamellipodial and filopodial cell protrusions, which are the key cellular structures for environmental guidance and cell migration, and play roles in cell transformation and cell motility [2]. Fascin is normally expressed in vascular endothelial cells and fibroblasts [6], and it has been reported that fascin is usually present at low levels in stratified basaloid squamous cells of the esophagus [9]. Previous data from a number of laboratories have highlighted that fascin is up-regulated in many human carcinomas and, in individual tissues, it correlates with the clinical aggressiveness of the tumor and with poor patient survival [4, 7, 11, 12, 18, 19, 24, 35]. These findings indicate that fascin may functionally contribute to disease progression [11].

Chen *et al.* reported that expression of fascin was up-regulated in most of their series of OSCC, though variability and heterogeneity in the intensity and distribution of fascin expression were present [4]. In other reports, intense staining of fascin protein was identified at the invasive fronts or infiltrating borders of most cases of OSCC, and overexpression of fascin was found to contribute to a more aggressive OSCC [4, 18]. In agreement with the previous reports, all of the present cases with invasive OSCC showed overexpression of fascin mainly in the cytoplasm of the tumor cells invading into the lamina propria. Fascin expression extended from the invasive fronts to the upper layer of the epithelium.

Previous reports on fascin have mostly focused on the fascin expression in human carcinomas [4, 7, 11, 12, 18, 19, 24, 35]. But in the current study, we focused on when the up-regulation of fascin occurred during the process of

malignant transformation and development of neoplasm. In previous studies, fascin overexpression has been observed in precancerous lesions or CIS, dysplasia of the esophagus [37], sinonasal inverted papilloma with dysplasia [30], adenoma of the colorectum [10], and intraductal papillary mucinous adenoma or borderline neoplasm of the pancreas [34]. Further, Yamaguchi *et al.* showed that fascin overexpression in intraductal papillary mucinous neoplasms of the pancreas were correlated with increased histological grade by immunohistochemical analysis [34]. Fascin may therefore contribute to the early stage of carcinogenesis. In this study, up-regulation of fascin was observed in oral dysplasia and CIS. To our knowledge, this is the first report that showed up-regulation of fascin appearing at an early stage of malignant transformation in oral lesions. Our research showed that fascin expression was not detectable in hyperplasia and the immunostaining scores for fascin were significantly higher in dysplasia and CIS than in benign disease. In all cases of invasive OSCC, overexpression of fascin extended from the destruction of the basal layer of the epithelium to the upper layer of the epithelium. This finding supported the reports of Zhang *et al.* that the level of fascin expression and frequency of overexpression increased gradually in the progression from normal epithelium to simple hyperplasia, dysplasia, CIS, and finally invasive esophageal squamous cell carcinoma [37].

It is reported that approximately 90% of OSCCs express podoplanin, which is restricted to the invasive front of the tumor, and high expression levels are associated with lymph node metastasis and a poor clinical outcome [16, 36]. In agreement with previous reports, our research showed podoplanin was overexpressed in the cytoplasm and membrane of the tumor cells invading into the lamina propria in all cases of invasive OSCC. Several studies showed that podoplanin remodeled the actin cytoskeleton of tumor cells, contributing to their increased migration and invasion [20, 22, 33]. The association of podoplanin with the actin-cytoskeleton was mediated by an ERM protein called ezrin. Ezrin was markedly phosphorylated and it induced remodeling of the actin cytoskeleton, thereby contributing to migration and invasion of cancer cells, when podoplanin was over-expressed. In addition, it has been reported that podoplanin modulates the activities of Rho GTPases, mainly RhoA, by inducing reorganization of the actin cytoskeleton [20, 33].

In intraepithelial neoplasia/borderline diseases, reports have revealed that podoplanin is highly expressed in the basal cell layers in some of the hyperplastic and dysplastic areas adjacent to the SCC [36]. Kawaguchi *et al.* showed that 37% of OL lesions diagnosed with hyperplasia or dysplasia histopathologically, exhibited podoplanin expression patterns similar to those found in OSCC immunohistochemically. They suggested that patients with podoplanin expressed lesions had a significantly higher incidence of invasive oral cancer, therefore podoplanin was a promising biomarker for predicting the risk of oral cancer development [14]. Moreover, it was reported that podoplanin was

abnormally expressed in lichen planus, which the WHO classifies as being a potentially malignant disorder, with the malignant transformation risk being much greater in patients with podoplanin over-expression [28]. These data highlight the importance of podoplanin detection in early oral tumorigenesis, suggesting that podoplanin may be used as a biomarker for risk assessment of malignant transformation in patients with potentially malignant oral disorders.

In the WHO criteria [5], the term dysplasia applies when architectural disturbance is histopathologically accompanied by cytological atypia (variations in the size and shape of the keratinocytes). However, oral benign lesions other than dysplasia also frequently show architectural disturbances and cytological atypia, which include reactive atypia known as inflammatory atypia and regenerative atypia, therefore it is often difficult to distinguish between dysplasia and other benign lesions. In our study, the immunostaining scores for fascin and podoplanin were significantly higher in dysplasia and CIS than in benign disease, and they were significantly higher in dysplasia than in benign disease. These findings suggest that oral dysplasia and CIS could be distinguished from other oral lesions by assessing the expression of fascin and podoplanin immunohistochemically, and that such assessment could improve the diagnostic accuracy of oral dysplasia and CIS.

In summary, this is the first report of an up-regulation of fascin occurring early in the progression from normal epithelium to invasive OSCC. Our results demonstrated that it was helpful for improving the diagnostic accuracy of oral dysplasia and CIS to assess the expression of fascin and podoplanin immunohistochemically.

A summary of this work was presented at the 33rd Annual Meeting of the Japan Society for Head and Neck Cancer (June 11, 2009, Tokyo).

V. Conflict of Interest Statement

None declared.

VI. References

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