Wnt5a Overexpression in Thick Primary Oral Mucosal Melanomas: Implications for its Role in Tumor Progression

Jing Xiao1, 2), Hitoshi Nagatsuka1), Chong Huat Siar3), Kok Han Ng4), Ryo Tamamura1), Rosario Santos Rivera1), Naoki Katase1), Masashi Inoue6), Kojun Setsu6), Keisuke Nakano5), Toshiyuki Kawakami5) and Noriyuki Nagai 1)

1) Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry & Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8525, Japan
2) Department of Oral Biology, College of Stomatology, Dalian Medical University, 465 Zhongshan Road, Dalian 116027, China
3) Department of Oral Pathology, Oral Medicine & Periodontology, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia
4) Unit of Stomatology, Cancer Research Centre, Institute for Medical Research, Jalan Pahang, 50500 Kuala Lumpur, Malaysia
5) Matsumoto Dental University Institute for Oral Science, Shiojiri, 399-0781, Japan
6) Laboratories for Structure and Function Research, Faculty of Pharmaceutical Science, Tokushima Bunri University, Tokushima 770-8514 Japan

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Abstract: Wnt genes encode a large family of secreted cysteine-rich signaling molecules involved in cell growth, differentiation and tumorigenesis. Wnt5a, a non-transforming member of the Wnt family behaves as a putative oncogene in many cancers including melanomas. The aim of our study was to determine Wnt5a expression in primary oral mucosal melanomas (OMM) and correlate it with tumor thickness. Archival tissues from 18 OMM cases were subjected to immunohistochemical detection of Wnt5a by the streptavidin-biotin method. These were categorized into tumors of <4 mm (thin and intermediate thickness lesions) and >4 mm (thick lesions) thickness. Most OMM cases (17/18; 94.4%) stained positive for Wnt5a, though heterogeneously. Seven thick (7/11; 64%) and one intermediate thickness (1/7; 14%) OMM demonstrated strongly positive Wnt5a staining (P<0.05). The only Wnt5a-negative case was a thick OMM without local recurrence after treatment. Strong Wnt5a expression at tumor advancing sites suggests a role in local tumor spread. Identification of pleomorphic epithelioid and spindle cells as melanoma cell populations with the most pronounced Wnt5a staining suggests that Wnt5a overexpression influences cellular phenotype. These results taken together suggest that Wnt5a is up-regulated in OMM and may play a role in tumor progression.

Key words: Wnt5a, Oral mucosal melanoma, Immunohistochemistry, Tumor thickness
In the latter, Wnt5a is of especial importance because microarray analysis has identified it as a gene that separates highly aggressive melanomas from their less invasive counterparts. Furthermore, a fivefold increase in Wnt5a mRNA level is seen in up to 50% of primary malignant melanomas while transfection of Wnt5a cDNA into non-invasive melanoma cells results in increased invasive potential. All these studies implicate that Wnt5a behaves as a putative oncogene in melanoma.

While molecular and other in vitro studies have contributed substantial knowledge and understanding on the oncogenic function of Wnt5a in melanomas, less is known about the immunohistochemical distribution of this molecule in human melanoma. A review of the English language literature disclosed that Wnt5a expression in OMM has not been previously investigated. The focus of this study was to examine by immunohistochemistry 18 cases of primary OMM for Wnt5a expression and to evaluate its correlation with tumor thickness. A possible role of Wnt5a in the neoplastic progression of OMM was also speculated.

### Materials and Methods

#### Tissue samples

The tissue samples in this study were from the surgical pathology files of the Department of Oral Pathology, Graduate School of Medicine and Dentistry, Okayama University, Japan, and Unit of Stomatology, Cancer Research Center, Institute for Medical Research, Kuala Lumpur, Malaysia. Clinical database variables including age, gender and tumor location of these cases were recorded. Information on tumor thickness (measured in millimeters from the most superficial layer of the oral stratified squamous epithelium to the deepest tumor cell) was extracted from pathology reports and clinical case notes. OMM cases were sorted into two categories with breakpoints set at <4 mm level (thin primaries: <1mm; and intermediate thickness lesions: 1.01 – 4 mm) and >4 mm level (thick lesions) for correlative studies with Wnt5a expression level.

Archival formalin-fixed, paraffin-embedded tissue blocks of 18 cases of histologically confirmed primary OMM were retrieved. New 3-ìm thick sections were prepared for staining with hematoxylin-eosin, and for immunohistochemistry with Wnt5a antibody [staining with melanoma markers including Masson-Fontana, S-100 protein, HMB-45 and Melan-A were performed previously for purposes of histological diagnosis of these tumors, but these data are not included here].

### Immunohistochemistry

The streptavidin-biotin method was used for the immunohistochemical detection of Wnt5a. Briefly, deparaffinized sections of 3-im thickness were pretreated for antigen retrieval by autoclave heating (132°C, 6 min) in 10 nM of citrate buffer.
These sections were then immersed in 0.3% methanol containing 1% hydrogen peroxide for 30 min, to block endogenous peroxidase, and rinsed in 0.05 M Tris-buffered saline (TBS) (5 min, three times) before immersing in blocking solution (Funakoshi, Japan) for 10 min at room temperature. Then the sections were covered with primary antibody (goat polyclonal anti-mouse Wnt5a at 1:25 dilution; R&D Systems Inc., Minneapolis, MN, USA) and incubated overnight at 4°C. Immunoreactions were performed using Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). The antigenic sites were visualized using 3-amino-9-ethylcarbazole (AEC) substrate chromogen (Dako, Carpinteria, CA, USA) and counterstained with Mayer’s hematoxylin. For negative control, sections were treated as above but without the primary antibody. All the control sections were negative. Positive staining control was also included and, where present in the specimens, internal staining controls were also checked for appropriate reactions with the primary antibody.

Figure 1. Advanced invasive OMM (A). Representative sections showing strong Wnt5a staining at advancing tumor sites: in and around epithelioid tumor nests (B-D), interconnecting cords of pleomorphic epithelioid cells (B, C), irregular islands (E) and sheets of pleomorphic epithelioid cells (F), and tumor prolongations composed of loosely cohesive spindle cells at the deep advancing front in the submucosa (G, H). Note that Wnt5a expression is localized at the cell membrane and cytoplasm of these melanoma cells (H). Insets depict OMM cases that stained weakly positive for Wnt5a (B, C). (A, H&E; B-H, Wnt5a; A,B, inset, x 40; c, inset, x200; D-F, H, x400; G, x 100).
Immunohistochemical scoring
The tumors were analyzed subjectively according to the proportion of immunoreactive tumor cells and intensity of Wnt5a staining, and were categorized as follows: negative, no staining of tumor; weakly positive, staining is faintly present in focal areas (<25%); moderately positive, staining is evident in large parts of tumor (25-50%); and strongly positive, pronounced staining is present in large parts of the tumor (>50%).

Statistical analysis
The Kruskal-Wallis and Mann-Whitney tests were performed to compare the expression level of Wnt5a with the clinical data of OMM including tumor thickness. P<0.05 was considered significant.

Results
Clinicopathological findings
The clinical variables of the 18 OMM cases are summarized in Table 1. There were 11 (61%) male and seven (39%) female patients. Their average age was 57.8 years (age range: 24-82 years). The tumor sites of origin in decreasing order of frequency were the upper gingival and palatal mucosa in 11 cases (61%) followed by the lower gingival mucosa in six cases (33%) and buccal mucosa in one case (6%).

According to the WESTOP classification of OMM (29), the sample studied comprised 18 invasive OMM, 13 with in-situ components. Fourteen (78%) OMM in this series were melanotic, the remaining amelanotic (Table 1). Histologically, the tumor cell morphology was predominantly mixed (spindle and pleomorphic epithelioid) in 12 (67%) tumors, pleomorphic epithelioid in four (22%) tumors, and spindle in two (11%) tumors.

Immunohistochemical findings
Wnt5a staining in OMM was heterogeneous: one tumor was negative, four were weakly positive (Fig. 1B, C inset), five displayed moderately positive staining and eight had large areas of strongly positive staining (Fig. 1A-H). In the latter two groups, the expression pattern was especially marked around pleomorphic epithelioid tumor nests (Fig. 1B-D), interlacing cords of pleomorphic epithelioid cells (Fig. 1B, C), islands (Fig. 1E) and diffuse sheets of pleomorphic epithelioid cells (Fig. 1F), and tumor prolongations composed of loosely cohesive spindle-shaped cells at the deep advancing front in the submucosa (Fig 1G, H). Positive immunoreactivity for Wnt5a was distinctly identified at both the...
cell membrane and within the cytoplasm of these melanoma cells (Fig. 1H). In these Wnt5a positive tumor areas, sometimes concomitant mild to heavy melanin pigment deposits may be present and these may mask the cellular details.

In those tumors that showed moderately to strongly positive Wnt5a staining, this distribution pattern extended inconstantly to the peripheral regions of the specimens where a predominant radial growth pattern was observed (Fig. 2A-F), as well as into those areas where the neoplasm was limited to the oral epithelium and epithelial-connective tissue interface (in-situ OMM components) (Fig. 3A-F). In the former, tumor nests (Fig. 2B, C) and diffuse spindle cell tumor sheets (Fig. 2D-F) displayed strongly positive staining for Wnt5a.

In in-situ OMM, Wnt5a expression was also heterogeneous: negative in some areas and positive in others. The Wnt5a-positive areas were usually found in single (Fig. 3 A, B) or scattered nests/clusters of large, pleomorphic and atypical melanocytes present at the epithelial-connective tissue junction (Fig. 3C-F).

### Correlation between Wnt5a expression level and tumor thickness

The results of Wnt5a expression level and tumor thickness are summarized in Table 2 and shown in Fig. 4. There were 11 (61%) thick and seven (39%) intermediate thickness OMM but no thin primaries. Their overall mean tumor thickness was 5.76 mm (range: 1.2 - 10 mm). A statistically significant association was observed between the expression level of Wnt5a and OMM tumor thickness. Seven thick OMM cases (7/11; 64%) compared with one intermediate thickness OMM (1/7, 14%) demonstrated strongly positive Wnt5a staining (P<0.05) (Fig. 4).

No statistically significant correlation was found between Wnt5a expression and the other clinicopathological variables of OMM (Table 2).

### Discussion

This study highlights for the first time the expression pattern of Wnt5a in primary OMM. Our results demonstrated that Wnt5a expression was up-regulated in 17 out of 18 cases (94.4%) of OMM. In the current literature, aberrant levels of Wnt5a has been reported in 10 primary melanomas and 24 melanomas but it is not known whether any of these tumors evaluated were of oral
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Overexpression of Wnt5a in OMM mucosal origin. Wnt5a overexpression has also been reported in other cancer types including those from the lungs, prostate and breast\(^{20,21}\), and in head and neck squamous carcinoma cell lines (30). Despite the observation of Wnt5a in many cancer types, no direct information is available on its expression in human OMM until now.

In this study, to assess the association between levels of Wnt5a expression and tumor thickness, OMM cases were sorted into two categories: tumors of \(<4\) mm (thin primaries: \(<1\) mm, and intermediate thickness lesions: \(1.01 – 4\) mm) and \(>4\) mm (thick lesions). There were 11 (61%) thick and seven (39%) intermediate thickness lesions, but no thin primaries. Our observations suggest a significant correlation between Wnt5a expression level and tumor thickness. Seven thick (7/11; 64%) compared with one intermediate thickness tumor (1/7, 14%) demonstrated strongly positive Wnt5a staining (P<0.05). This finding suggests that very high levels of Wnt5a activity may frequently be acquired late in OMM development, thereby implicating this molecule in tumor progression. In some cancers including melanomas, early expression of Wnt5a may result in suppression of tumorigenesis, whereas if it is expressed at a later stage, it becomes a potent inducer of cancer cell migration and motility\(^{19}\). As only a limited number of OMM were evaluated here, studies on larger series including thin primaries are needed.

Table 2 Wnt5a expression and clinicopathological characteristics of 18 patients with primary oral mucosal melanomas

<table>
<thead>
<tr>
<th>Intensity of Wnt5a staining</th>
<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
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<td>3</td>
<td>4</td>
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<td>(\leq4) mm</td>
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<td>3</td>
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<tr>
<td>(&gt;4) mm</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>7</td>
<td>11</td>
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(-): negative, (+): weakly positive, (++): moderately positive, (+++): strongly positive; * In three cases, the tumor involved both the palatal and gingival mucosa.

Figure 4. Level of Wnt5a expression correlates with oral melanoma thickness of progression.

Figure 4: Level of Wnt5a expression correlates with oral melanoma thickness of progression (\(-\): negative, \((+\)) +: weakly positive, \((++\)) ++: moderately positive, \((+++\)) +++: strongly positive; ** Fp=0.01, * 0.05•significance by chi-square test.

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to clarify the clinical relevance of this observation more fully.

The heterogeneous pattern of Wnt5a staining in OMM as observed in this study correlated well with those in primary melanomas from other sites[26,27]. Reportedly, melanomas tend to show a wider gamut of staining for Wnt5a compared to nevi[27], and have a more variable expression level[26]. This inconsistent staining pattern reflects the heterogeneity of Wnt5a activity in different parts of a given tumor[25,27]. In cancers of lungs, prostate and breast the immunohistochemical pattern of Wnt5a expression is unclear[20,21].

In melanomas, cell populations with strong Wnt5a expression become more pronounced in higher-grade tumors[27]. In our series, 17/18 OMM were considered high-grade tumors based upon an integrated assessment of their histomorphological features in HE stained sections, their clinical course and Wnt5a expression level. Clinically, all cases presented with progressive disease manifested as regional lymph node metastases, and all but one case (Wnt5a-negative tumor) developed local recurrence after primary treatment. This single Wnt5a-negative OMM was considered low-grade. It has also been suggested that Wnt5a expression level correlates with both the survival and time to the development of metastases[27]. We have no follow-up data of our present series to confirm this contention.

There are conflicting reports concerning correlation between intensity of Wnt5a expression and morphological forms of melanoma cells. One study demonstrated that melanoma cells with giant cell and sarcomatoid morphology tend to stain strongly positive for Wnt5a compared to epithelioid cell forms, and suggested that the two former cell types are highly malignant cellular phenotypes associated with aggressive melanomas[27]. Another report found that Wnt5a expression was strongly positive in melanomas characterized by small uniform melanoma cells, and weakly positive in those showing large pleomorphic melanoma cells[26]. The one hundred month survival for melanomas with small epithelioid cells is 49%[31]. In the present study, the main tumor cell morphology encountered were pleomorphic epithelioid and spindle cells. Parallel microscopic examination of HE stained sections, their clinical course and Wnt5a expression level of each tumor was methodically performed in an attempt to relate Wnt5a-positive areas with the cell types observed. We found that in Wnt5a-positive OMM, both pleomorphic epithelioid and spindle cells showed comparable Wnt5a staining within a given tumor. These observations suggest that in OMM, these two cell types most probably represent melanoma cell populations associated with tumors of a more aggressive behavior. Furthermore, the observation that these Wnt5a-positive cells occurred at sites of the tumor corresponding to the radial and vertical growth phases of OMM suggest that Wnt5a activity may relate to both growth phases. The disparity in findings between our study and previous reports suggests that there may be some fundamental differences governing the pathobiology of OMM and melanoma at other sites.

In summary, the results of our study presented herein support the following key observations: 1. A high proportion of OMM tend to show up-regulation of Wnt5a expression, though heterogeneously; 2. Wnt5a activity is most frequently detected in advanced OMM where it may play a role in tumor progression; 3. expression level of Wnt5a in OMM correlates with tumor grade, thickness and cellular morphology; and 4. further investigations are needed to explore the potential value of Wnt5a as a therapeutic target in OMM.

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References

17. Goodwin AM, D’Amore PA. Wnt signaling in the vasculature. Angiogenesis 5:1-9, 2002