I. Introduction

Mucosal melanoma is an aggressive neoplasm that warrants separate consideration from cutaneous melanoma. Even small lesions of mucosal melanoma behave aggressively with high rates of recurrence and death. To reflect this aggressive behavior, 2010 AJCC (American joint committee on cancer) announced (1) that primary melanomas limited to the mucosa of the head and neck are classified as T3 lesions. This is due to the advanced nature of the disease at the time of initial diagnosis, rich vascular and lymphatic supply of mucosal sites, and the lack of clinical suspicion due to the rarity of these tumors. Oral melanoma begins as a darkly pigmented, irregularly marginated macule, which later develops a nodular invasive growth phase. However, it is often nodular at the time of diagnosis, but early lesions may be flat (2). They can send metastatic deposits after a latent period of 2-20 years to several sites, such as lymph nodes, bone, lungs, liver, gastrointestinal tract, and subcutaneous tissue (3). In most instances, melanoma cells contain fine melanin granules, but a lack of melanin production may cause diagnostic confusion because melanoma can mimic a variety of undifferentiated tumors. Immunoreactivities for S-100, HMB-45, Melan-A, and Mart-1 are beneficial in distinguishing such melanomas from other malignancies.

Melan-A and Mart-1 molecules have the evidence sum classified as concordant where eligible and independent studies of these molecules have generally agreed with each other in terms of significance and direction of the effect (4). However, in HMB-45 (gp100/Pmel17 molecule), the results of independent studies were discordant; the results of immunohistochemistry-based analysis have disagreed with both the significance and the direction of prognostic factor. HMB-45, originally produced by Gown and Vogel et al in the 1980s, defines an 'oncofetal' premelansomal antigen that is positive on fetal melanocytes and melanoma cells, but negative on adult resting melanocytes (5).

Nestin immunoreactivity was observed in HMB45-negative melanoma cells in the dermal parts of all cases of amelanotic and melanotic nodular melanomas, but Nestin expression was negative in 10 of 12 cases of superficial spreading melanoma (6). We performed the present study in order to solve the question regarding not only the relationship between HMB-45 and Nestin but also the value of prognosis factors. Nestin is an intermediate

filament protein which serves as a hair follicle stem cell and a neural stem cell marker. Recent studies have suggested that Nestin expression is also important for tumorigenesis. Klein et al observed a staining increase in primary and metastatic melanomas with respect to percentage of cells and staining intensity (7). When Nestin expression and depth of invasion were evaluated, strong expression predominated the lesions with dermal invasion over 1mm (8). Nestin-positive cases in all anatomic stages showed a significant decrease of 5-year survival rate and it was suggested that Nestin expression could be a predictor of poor prognosis in patients with cutaneous malignant melanoma (9). Furthermore, Piras et al. suggested that Nestin expression in both tumoral and endothelial cells may be considered an important early prognostic marker for melanoma (10).

Therefore, in the present study, we examined the prognostic significance of Nestin for stage advance of oral malignant melanoma, and investigated the relationship among the expression patterns of Nestin and HMB-45, the time to regional lymph node

metastasis and distant metastasis, depth of invasion, and angiogenesis.

II. Materials and Methods

Patients and tissue specimens

Archival tissue blocks from 39 patients, 6 melanoma in situ and 33 malignant melanoma with vertical invasion, who underwent observation at the Department of Oral Pathology, School of Dentistry, Seoul National University Dental hospital, Seoul, South Korea, between March 1999 and October 2012, were selected for this study. The clinical stage, pathological anatomic stage, invasion depth, and prognosis after surgery were considered in a total of 39 evident examples. Age, gender, tumor location, adjuvant therapy, time from finding the mucosa-pigmentation to first diagnosis and the time of finding the regional lymph node and distant metastasis were included into chart analysis factors. Clinical data, obtained until October 2012, were available for 11 patients with stage III, 19 patients with stage IVA and 9 patients with stage IVC melanoma, accordingly to the 2010 AJCC Staging System. Similar to the anatomic stage of cutaneous melanoma (1), we also classified stage IVA into two phases, based on the presence of lymph node metastases, to evaluate the prognoses of 5 total classifications. Among 39 total cases including 6 melanoma in situ cases, we have 32 and 30 cases of the tissue blocks from initial biopsy which showed radial growth area and vertical growth

area, respectively. In these patients, we investigated the various scales of Nestin staining with respect to distant metastasis-free survival(DFS) and stage advance-free survival(SFS) time. Regional lymph node metastasis was found in 12 patients on their first hospital visit. One patient developed distant metastasis without regional lymph node metastasis. Therefore, twenty six patients showed no lymph node metastases on the initial diagnosis, and we were able to follow up on regional lymph node metastasis-free survival(RFS) time in these patients. The study protocol was approved by Seoul National University Dental Hospital Institutional Review Board.

Immunohistochemistry

Serial microtome sections 5㎛ thick were treated immunohistochemically for Nestin and HMB-45. Heat induced antigen retrieval was performed at 95°C for 10 min in target retrieval solution (Dako, pH6.0), for Nestin and HMB-45 antigen. Mouse monoclonal antibodies against Nestin (MAB5326; Millipore Corp, Schwalbach/Ts., Germany; 1:200 dilution) and human HMB-45 (clone HMB-45; Dako; 1:100 dilution) were used as primary antibody. Polymer-HRP EnVision detection system (DakoCytomation, Denmark) was used as secondary antibody. We used anti-human antibodies against Nestin at an incubation time of

20 hours. For HMB-45, we incubated the sections for 1 hour. Finally, immunoreactive proteins were visualized with NovaRED (VECTOR) and counterstained with Mayer' hematoxylin. Human tissue specimens, which strongly expressed Nestin were used as positive controls. Independent histopathological analysis was performed by two pathologists.

Staining evaluation

Invasion depth was categorized into four groups as follows; melanoma in situ -> 0, ≤4mm -> 1, 4.01-8.0mm -> 2, >8.0mm -> 3. In radial growth area of malignant melanoma, Nestin positive staining was quantitatively interpreted as follows; no expression -> 0, focal or <15% -> 1, diffuse or >15% -> 2. In vertical growth area, two quantitative methods for the Nestin staining level were evaluated as follows. The first is the measuring of Nestin staining level at the vertical tumor nest, which was made in proportion, and the second is to compare the amount of positive cells in Nestin and HMB-45. In the former, the absolute level of Nestin staining was assessed by the proportion of stained cells in vertical tumor nests, using a 1 to 3 scale for expression as follows; 1-33% -> 1, 34-66% -> 2, 67-99% -> 3. In the latter, the relative level of Nestin staining was assessed by the amount of Nestin positive cells, compared to the amount of HMB-45 positive cells, using a 1

to 3 scale for expression as follows; Nestin(+) cells less than HMB-45(+) cells -> 1, Nestin(+) cells almost same amount of HMB-45(+) cells ->2 , Nestin(+) cells more than HMB-45(+) cells -> 3. Intensity of Nestin was defined and categorized into four groups, according to the relative staining intensity of Nestin of the tumor cells, compared with that of the vascular endothelial cells around tumor nest (9) as follows; no expression -> 0, weaker than endothelial cells ->1, same as endothelial cells -> 2, and stronger than endothelial cells -> 3.

Analysis of clinical parameters

The correlation analysis with Spearman correlation coefficient was performed to investigate the relationship between various clinical variables and various scales of Nestin staining on an initial diagnosis. Survival analysis was performed, using Kaplan- Meier method (additionally log-rank test to compare curves) and Cox proportional hazards regression analysis, in order to investigate the regional lymph node metastases-free survival(RFS), distant metastasis-free survival(DFS), and stage advance-free survival(SFS). Statistical significance was defined as a two sided P-value <0.05. The statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS software Version 12.0, SPSS Inc., Chicago, IL, USA).

III. Results

Clinical features

Among the 39 total cases, affected persons were usually between the age of 45 and 69 years(Mean±SD: 57.1±13.1). Males were more affected by a ratio of 20:19. In terms of the location, 33 cases(84.6%) occurred in the maxilla, including the hard palate or the maxillary alveolus, and the remaining 6 cases(15.4%) in the mandible(7.7%: 3 cases), lip(5.1%: 2 cases), and buccal mucosa(2.6%: 1 case).

On the time basis of initial diagnosis, all three scales on Nestin staining in the vertical growth area of primary melanoma did not show significant correlation with anatomic stage, invasion depth, patients' age, and gender (Table 1). The staining level of Nestin in the radial growth area showed significant positive correlations with initial anatomic stage, invasion depth, and age ($p=0.002, \gamma=0.517$; $p=0.001, \gamma=0.559$; $p=0.045, \gamma=0.357$; respectively; Table 1).

We were able to obtain the clinical data about time from finding the mucosa-pigmentation to first diagnosis in 22 patients. The time from finding the mucosa-pigmentation to first diagnosis showed the significant inverse correlations with initial stage, invasion depth, and the level of Nestin staining manifestation in

the radial growth area (p=0.024, γ = −0.478; p=0.005, γ = −0.573 ; and p=0.009 , γ = −0.544, respectively; Table 2).

Radial growth lesion

Among all cases of oral malignant melanoma, 6 cases were diagnosed as melanoma in situ. Nestin staining on tissues from various parts of the 6 cases indicated that 5 cases were Nestin−negative and only one case was Nestin−positive. In this Nestin−positive case, a significant amount of melanophage was found in the lamina propria of the oral mucosa. The Nestin staining in radial growth area indicated more positivity in stage IV (100%:21/21) than in stage III (27%:3/11). In the three cases of stage III, which showed a Nestin positive staining in the radial growth area, there was one recurred case after adjuvant therapy and the underlying connective tissue was invaded.

As explained in the methods section, among the 26 patients who showed no signs of regional lymph node metastases on their first hospital visit, 24 cases of our own tissue blocks from initial biopsy showed radial growth areas. The staining level of Nestin in the radial growth lesion had the significant effect on 5−year RFS (log−rank test, p=0.029), while not showing any significant effect on 5−year DFS, 5−year SFS on anatomic stages of mucosal

melanoma of 2010 AJCC and 5-year SFS on the classification of stages similar to cutaneous melanoma (log-rank test, p>0.05).

Vertical growth lesion

The stained images of Nestin and HMB-45 in the vertical growth area of oral malignant melanoma are shown in figure 1. Among the 26 cases with RFS time as already mentioned, with exceptions of 6 melanoma in situ cases, 19 cases of our own tissue blocks from initial biopsy showed vertical growth areas. Melanoma's depth of invasion was associated with 5-year RFS (log-rank test, p=0.003; Fig. 2a). Intensity of Nestin in the vertical growth area which is compared to the staining level of blood vessel showed no correlation with 5-year RFS (p=0.328; Fig. 2b). Analytical results showed that absolute staining levels of Nestin was not associated with 5-year RFS (log-rank test, p=0.596; Fig. 2c), whereas the relative staining levels of Nestin was associated with 5-year RFS (log-rank test, p=0.016; Fig. 2d).

Kaplan- Meier survival analysis on distance metastasis and stage advance of the initial biopsy in the vertical growth area from 30 patients showed the following: The absolute degree of Nestin staining in the vertical growth area was not associated with 5-year DFS (p=0.211, log-rank test; Fig. 3c), whereas the relative

level of Nestin staining compared with HMB-45 staining in vertical growth was associated with 5-year DFS ($p<0.001$, log-rank test; Fig. 3d). Both the absolute and relative level of Nestin staining in vertical growth were associated with 5-year SFS according to anatomic stages of mucosal melanoma of 2010 AJCC($p=0.018$ and <0.001, respectively, log-rank test, Fig. 4a, b). However, only the relative degree of Nestin was associated with 5-year SFS according to the classification of stages similar to cutaneous melanoma ($p<0.001$, log-rank test, Fig. 4c, d). Nestin intensity in the vertical growth area was not associated with either 5-year DFS or SFS on the 2 different stage systems above mentioned. Melanoma's depth of invasion also showed the correlation with neither 5-year DFS nor SFS on the 2 different stage systems.

We performed Cox proportional hazard regression analysis using the prognostic factors including adjuvant therapy that might affect melanoma's clinical progress and the relative and absolute Nestin level associated with stage advance on 2010 AJCC by Kaplan-Meier survival analysis. However, the results indicated that no further models would be fitted because the coefficient did not

converge. In turn, using the three above prognostic factors, Cox proportional hazard regression analysis was performed for stage advance-free survival according to the classifications of stages similar to cutaneous melanoma (Table 3). Only the relative staining level of Nestin compared to HMB-45 was significantly correlated with stage advance-free survival on those classifications (level 2(compared to level 1): HR, 14.45; 95% CI, 1.09-191.58; P=0.043 and level 3(compared to level 1): HR, 69.22; CI, 4.08-1173.24; P=0.003).

IV. Discussion

In all our cases of oral malignant melanoma, the distributions by age, gender, and anatomic location are almost the same as that reported by Neville et al. (2) except for one finding. In that study, two thirds of patients were men. The affected population in our study had a higher proportion of women than that result, but men were more affected by a ratio of 20:19. When patients visit the hospital for the first time, the anatomic stage and invasion depth shows a correlation with Nestin staining in the radial growth area but not in the vertical growth area. As Brychtova et al. described (8), the reason was related to the increase in Nestin concentration which was frequently observed in the peripheral and invasive parts of melanoma. It is difficult to infer invasion behavior before the patient visits the hospital with cells of central part of vertical growth lesion which were already differentiated and lost the tendency of invasion.

The expression of Nestin in the radial growth area showed relevance with time to regional lymph node metastasis. As the reason is already generalized, the depth of malignant melanoma is the most important factor to determine the time to lymph node metastasis (11). Therefore, as mentioned before, the expression of Nestin in the radial growth area which had a correlation with the depth showed a significant relationship with lymph node metastasis. However, the expression of Nestin in the radial growth area was not related to distant metastasis because more factors were involved in distant metastasis. But in the vertical growth area, the relative level of Nestin staining comparison with HMB-45 had a significant association with regional lymph node metastasis, distant metastasis and stage advance. The invasion depth of oral malignant melanoma is the most important factor for evaluating the lymph node metastases. Here, the relative level of Nestin staining, in comparison with HMB-45, can be helpful.

In our study, the ratio of regional lymph node metastasis was confirmed as 41% (16/39) of oral malignant melanoma cases

through histological diagnosis, which was higher than that reported by Medina et al. (12). Since distant metastases had high relevance with circulating tumor cells in peripheral blood, the presence of circulating tumor cells has been included as a new item into tumor staging systems for several carcinomas (13). Although tumor cells spread from primary melanomas to metastatic deposits by both lymphatic and vascular routes, lymph node metastasis usually present earlier than hematogenous ones. In cutaneous melanoma, 2010 AJCC claimed that lymph node metastatic melanomas of volumes smaller than 0.2 mm were clinically significant enough to distinguish between stages I, II, and III (1). Accordingly, we classified stage IVA into two phases, based on presence of regional lymph node metastases, in order to evaluate the prognosis of 5 classifications.

As observed in the vertical growth area, the comparison of amount of cells expressed in Nestin and HMB-45 was discovered as a significant prognostic factor for regional lymph node metastasis, distant metastasis and stage advance rather than quantitative measurement of the staining percentage in melanoma tumor nest. As Sellheyer et al. described (14), Nestin showed positivity in undifferentiated stem cells rather than HMB-45 expressed in fetal melanocytes, and some cells could express both

Nestin and HMB-45 at the same time (Figure 5). They reported that Nestin stained follicular dendritic cells with the same distribution pattern of HMB45-positive melanocytes within the follicular epithelium but distinct from the distribution pattern of Langerhans cells. However, although HMB-45 also stained the dendritic cells in the interfollicular epidermis and the hair matrix, Nestin exclusively stained intrafollicular dendritic cells in the developing skin (14).

We would examine the basis for this in more detail. Melanosomes are a type of lysosome-related organelle that has the unique capacity to produce melanin pigment, and that they progress through four sequential morphological stages as they mature (15). The stage I melanosome is at a polypeptide biosynthesis stage, which involves amorphous, round, and membrane-bound vesicles. In stage II, the melanosome is elongated and has a fibrillar structure, which depends on correctly processed and mature Pmel17. Melanins are subsequently synthesized and deposited on those melanosomal fibers, at which time the organelles are termed stage III melanosomes. In highly pigmented tissues, they are termed stage IV melanosomes (16). HMB-45 reacts specifically with the fibrillar matrix in stage II melanosomes (17). It has recently been shown that HMB-45

reacts with the sialylated RPT domain (18). Therefore, we speculate that fetal melanocytes show HMB-45 positivity, because it is prior to the melanin synthesis stage and there is a significant production of premelanosome (stage II melanosome). On the other hand, the premelanosomes produced in adult melanocytes react with tyrosinase and transform into melanosomes (stage III and IV melanosomes). In turn, the minute number of premelanosomes is not detected by the HMB-45 marker, and we suggest that this can be the primary reason why HMB-45 staining is negative.

Whereas Nestin staining was positive in the radial growth area in 100% of stage IV cases, only 27% of the cases were positive in stage III. This can be explained with the aforementioned theory as follows. Although the malignant melanoma of the radial growth area in stage III is more differentiated than in the Nestin-positive stage, it is proposed that stage III melanoma could show HMB45-positive and Nestin-negative findings because premelanosome and tyrosinase do not engage in a normal reaction, which causes melanoma to possess a significant amount of the cytoplasmic premelanosomal glycoprotein gp100 (Pmel17) (A area of Figure 5). On the other hand, in the vertical growth area, more undifferentiated and dedifferentiated melanoma cells exist than in

the radial growth area, increasing the number of malignant melanoma cells whose development of correctly processed and mature Pmel17 (gp100) could be hindered, resulting in MHB45-negative and Nestin-positive findings (C area of Figure 5). In our opinion, this is why HMB-45 is stained in an arbitrary, patchy fashion in the vertical invasion area, and why it shows gradually weaker staining in the deeper vertical growth phase, as has been previously reported (19). We also suggest that our staining and statistical analysis results are related to the aforementioned hypothesis. In evaluating the expression rate of Nestin alone in the melanoma tumor nest, there was no significant result with distant metastasis and lymph node metastasis by the Kaplan-Meier survival analysis, because the evaluation included cells that were stained by Nestin and HMB-45 simultaneously (B area of Figure 5). However, as the number of cells stained by Nestin (B and C area of Figure 5) surpassed those stained by HMB-45 (A and B area of Figure 5), the malignancy of melanoma became elevated, accelerating the advancement of anatomic stage, distant metastasis and lymph node metastasis as demonstrated by the Kaplan-Meier survival analysis.

In cutaneous melanoma, Nestin expression in both tumoral and endothelial cells was considered as an important prognostic

marker for melanoma (10). However, Kerr et al claimed that there was no association between microvascular density and particular clinical or pathological features of oral mucosal melanoma (20). Our results did not prove the association of Nestin positivity in endothelial cells with the prognosis of oral malignant melanoma. The reason was that the blood and lymphatic vessels were superficial in mucosa and closer to the site of extracutaneous melanoma (21). Since blood vessels were enriched and new blood vessels were heavily generated in the oral normal mucosa rather than in the skin, Nestin showed positivity in peripheral endothelial cells in almost all oral melanoma cases. Thus, similar to the method of Piras et al., we could not assess the relationship between the prognosis of melanoma and expression of Nestin in endothelial cells (10). With Nestin staining alone, it is difficult to identify the degree to which new blood vessels have been formed around normal mucosa and melanoma. However, Rivera et al claimed that oral melanoma cells caused the manifestation both of heparanase and of the most potent angiogenic mitogen: vascular endothelial growth factor (VEGF) (22). Their study notes that further research on angiogenesis is necessary.

When Nestin was found in the early 1990s, it was possible that the higher level of Nestin in the infiltrating parts might suggest a

role for Nestin in the tumor invasion (23). Nonetheless, it was reported that although Nestin was expressed in a slightly greater percentage of cases of melanoma than nevi and showed a greater intensity in melanoma, they found no statistically significant differences between nevi and melanoma (24). However, Sellheyer et al. claimed that only nevus was Nestin positive but not the adjacent residential melanocytes within the epidermal basal cell layer (14). Based on these results, we conclude that Nestin levels activate migrating melanocytes, not only malignant cells because a Nestin positive cytoplasmic network is more compatible with the structural dynamics of cell fluidity and mobility. Therefore, immunodetection of Nestin cannot be used to distinguish a benign lesion from a malignant lesion but that would be useful for the diagnosis of advanced-stage melanomas. In our study, there were Nestin-positive findings in 17% of melanoma in situ cases and in 91% of the radial growth areas of invasive melanomas. In the Nestin positive case of melanoma in situ, a significant amount of melanophage was found in the lamina propria. We presume that this

could be a protection process against micro-invasion. The invasion depth at vertical growth phase showed a significant correlation with the Nestin-positive level of the radial growth area (Table 1). Moreover, the time from the initial finding of pigmentation to the first diagnosis showed a significant correlation with the Nestin-positive level of the radial growth area and invasion depth (Table 2). From our findings, it can be inferred that Nestin-manifestation level is an important factor in the transition from the radial growth phase to the vertical growth phase. We believe that malignant melanoma cells gain fluidity and mobility through the Nestin-positive intermediate-filament protein of the cytoplasmic network; this allows the melanoma cells to transition from the radial to the vertical growth phase.

However, there is increasing evidence that cancer may arise from transformed stem cells. Hanahan et al. suggested that normal tissue stem cells might serve as the cells-of-origin that undergo oncogenic transformation (25). If we assume that melanocyte stem

cells are also involved in oral malignant melanoma, the following hypothesis can be established. The fact that 100% of malignant melanoma cases in the vertical growth phase were Nestin-positive (B and C area of Figure 5), in comparison with only 17% in melanoma in situ cases, contradicts the hypothesis that malignant melanoma of the radial growth phase will be differentiated from stem cells (C area of Figure 5). In the vertical growth phase, malignant melanoma cells are derived from melanocyte stem cells, resulting in the variety and abundance of Nestin-positive cells (B and C area of Figure 5). This raises suspicion that stem cells might not be involved in the radial growth phase. Consequently, we have to take into account the possibility that melanocyte stem cells might play an important role in the transition from the radial to the vertical phase. In turn, our estimation accords with the claim made by Girouard et al., in which melanoma is markedly resistant to therapy at more advanced stages, even though it is highly curable if identified during the radial growth phase (26).

In terms of the types of normal tissue stem cells involved in malignant melanoma, one of the primary candidates is epidermal melanocyte stem cells in the hair follicle, which are also intrafollicular Nestin-positive dendritic cells (27); however, there are no hair follicles in the oral mucosa. Another possibility is pigment cells originating from nerves, and we suggest that progenitor populations present along the nerves can supply normal melanocyte stem cells (28). Accordingly, it is proposed that dermis-derived stem cells, previously discussed by Zabierowski et al. (29), can be a source of epidermal melanocytes and melanoma. In our opinion, it is possible that these melanocyte stem cells derived from lamina propria and submucosa are involved in the transition from the radial to the vertical growth phase in oral malignant melanoma. Furthermore, our hypothesis is also supported by the fact that, among the four subtypes of cutaneous melanoma, acral lentiginous melanoma is the pathologic prototype of oral malignant melanoma (2).

V. Conclusion

It is suggested that we should evaluate the relative Nestin-staining expression compared with HMB-45 in oral malignant melanomas to predict the advancement of anatomic stage, distant metastasis, and regional lymph node metastasis. The invasion depth of the melanoma and the Nestin manifestation of the radial growth area are also important factors for predicting regional lymph node metastasis. We propose that Nestin manifestation can play an important role in the progression from the radial growth phase to the vertical growth phase, possibly because the Nestin cytoplasmic network is more compatible with the structural dynamics of fluidity and mobility. Furthermore, we postulate that dermis-driven stem cells, such as the progenitor populations present along the nerves, could act as factors in the transition from the radial growth phase to the vertical growth phase.

VI. References

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Tables

Table 1. Relationship between Nestin manifestation of oral mucosal melanoma and various clinicopathologic variables (n=39). The correlation analysis was indicated by the p-value and γ (spearman correlation coefficient), respectively.

Clinical Variables	Radial growth lesion	Vertical growth lesion		
	Nestin	Absolute Nestin	Relative Nestin (/HMB-45)	Intensity of Nestin
Initial stage	0.002*, 0.517	0.074, -0.331	0.060, 0.348	0.564, 0.110
Depth of invasion	0.001*, 0.559	0.102, -0.304	0.232, 0.225	0.833, 0.040
Age	0.045*, 0.357	0.134, 0.280	0.231, 0.225	0.393, -0.162
Gender	0.118, -0.282	0.338, 0.181	0.4, 0.159	0.338, 0.181

*p-value <0.05

Table 2. Relationship among the Nestin manifestation of oral mucosal melanoma, various clinicopathologic variables, and the time from finding the mucosa-pigmentation to initial diagnosis (n=22). The correlation analysis was indicated by the p-value and γ (spearman correlation coefficient), respectively.

	Time from finding the pigmentation to initial diagnosis
Initial stage	0.024*, -0.478
Depth of invasion	0.005*, -0.573
Age	0.585, -0.123
Gender	0.342, 0.213
Nestin in the radial growth area	0.009*, -0.544
Absolute Nestin†	0.293, 0.271
Relative Nestin(/HMB-45)†	0.325, 0.254
Intensity of Nestin†	0.166, -0.352

* p-value <0.05.

† Three parameters indicate the staining levels of Nestin in the vertical growth area.

Table 3. Multivariate Cox regression analysis for stage advance-free survival according to the classifications of stages similar to cutaneous melanoma (n= 30).

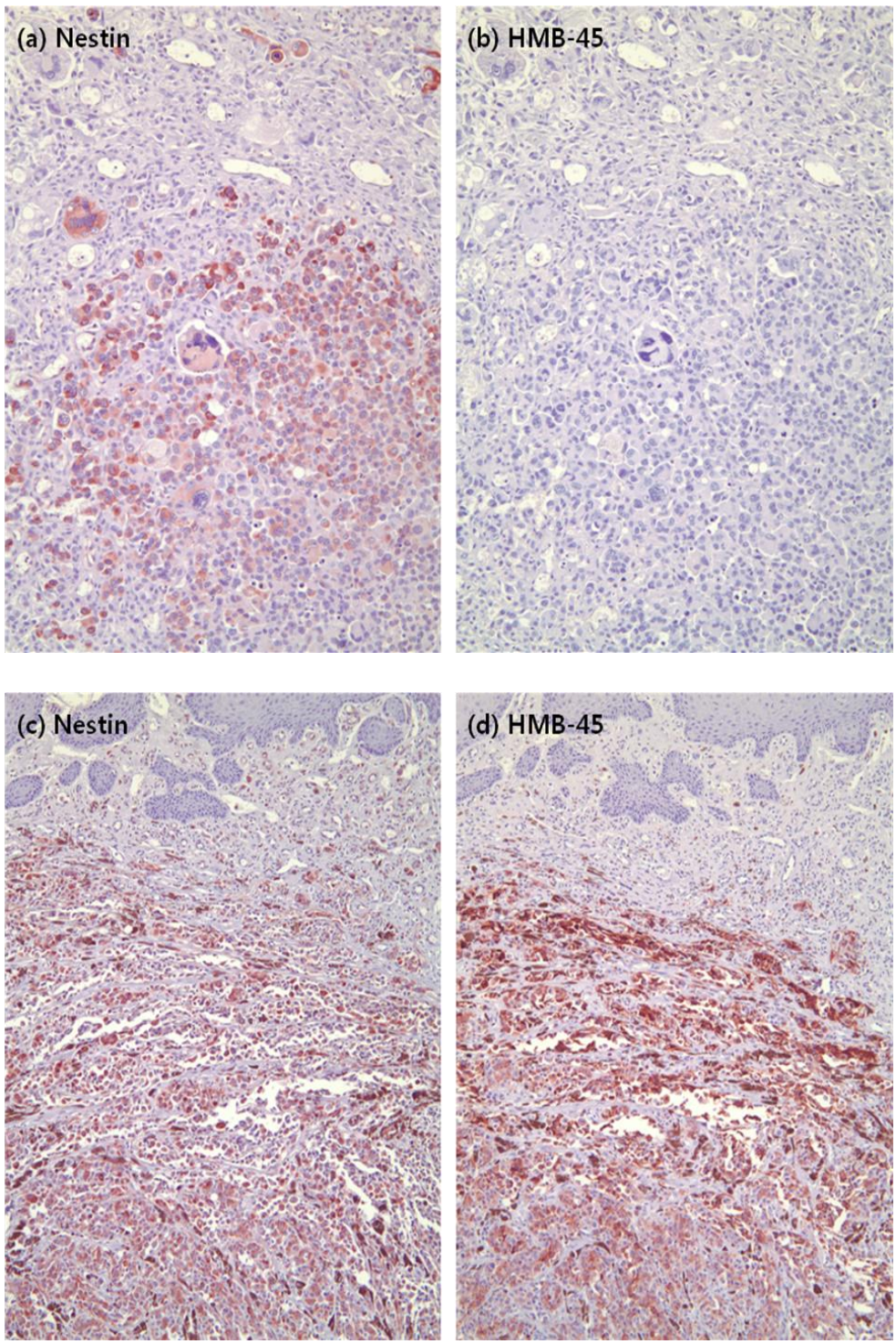
Variable		HR	95.0% CI	*P*
Relative Nestin(/HMB-45)				
in vertical growth area				
	level 1	1		
	level 2	14.45	1.09 to 191.58	0.043*
	level 3	69.22	4.08 to 1173.24	0.003*
Absolute Nestin				
in vertical growth area				
	level 1	1.00		
	level 2	0.97	0.13 to 7.23	0.975
	level 3	0.66	0.07 to 5.78	0.705
Adjuvant treatment		0.38	0.09 to 1.59	0.184

Abbreviations: HR, hazard ration; CI, confidence interval.

*p<0.05

Figures

Figure 1. Two representative cases show the discrepancy between the absolute level of Nestin staining and the relative level of Nestin compared with HMB-45 (a, c: Nestin; b, d: HMB-45). (a,b) The proportion of Nestin in overall melanoma nests showed level 1 of absolute Nestin. However, many pleomorphic and bizarre melanoma cells were Nestin-positive and HMB45-negative. Consequently, the comparison of the number of cells expressed in Nestin and HMB-45 yielded relative-Nestin level 3. (c,d) The proportion of Nestin in overall melanoma nests showed level 3 of absolute Nestin, but there was a higher level of manifestation in HMB-45 than in Nestin. Consequently, the comparison of the number of cells expressed in Nestin and HMB-45 yielded relative-Nestin level 1. Original magnifications: (a) to (d) X 100.


(a) Nestin
(b) HMB-45
(c) Nestin
(d) HMB-45

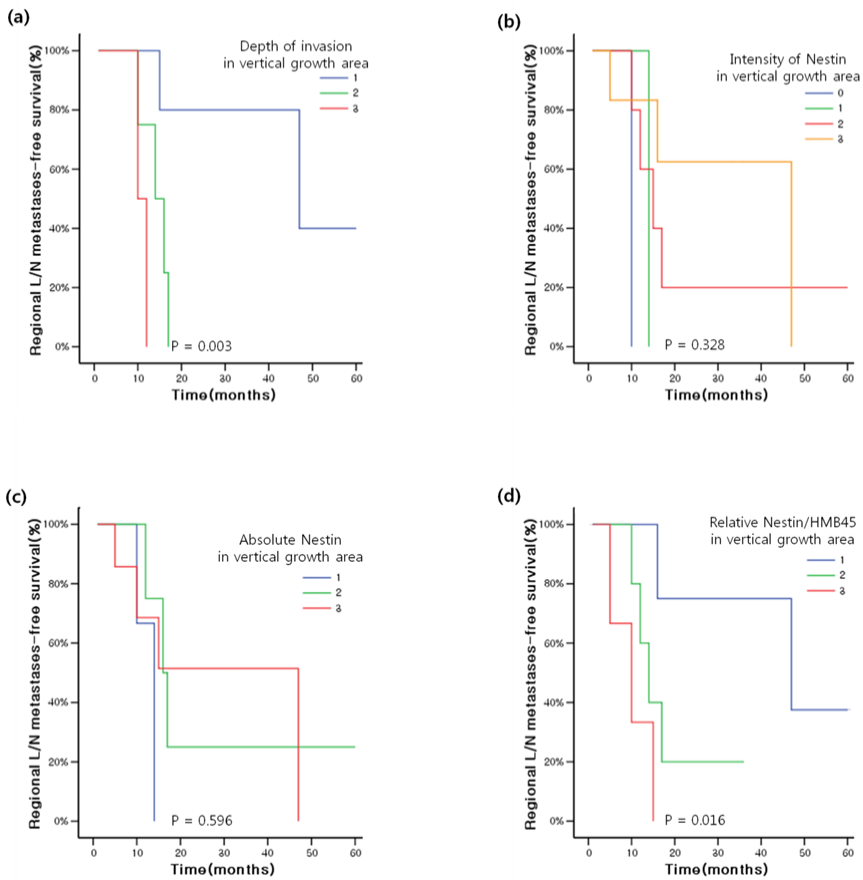


Figure 2. Kaplan-Meier curves for regional lymph node metastases of oral malignant melanoma according to (a) depth of invasion in the vertical growth area, (b) intensity of Nestin, (c) absolute staining level of Nestin, and (d) relative staining level of Nestin compared to HMB-45 (n=19).

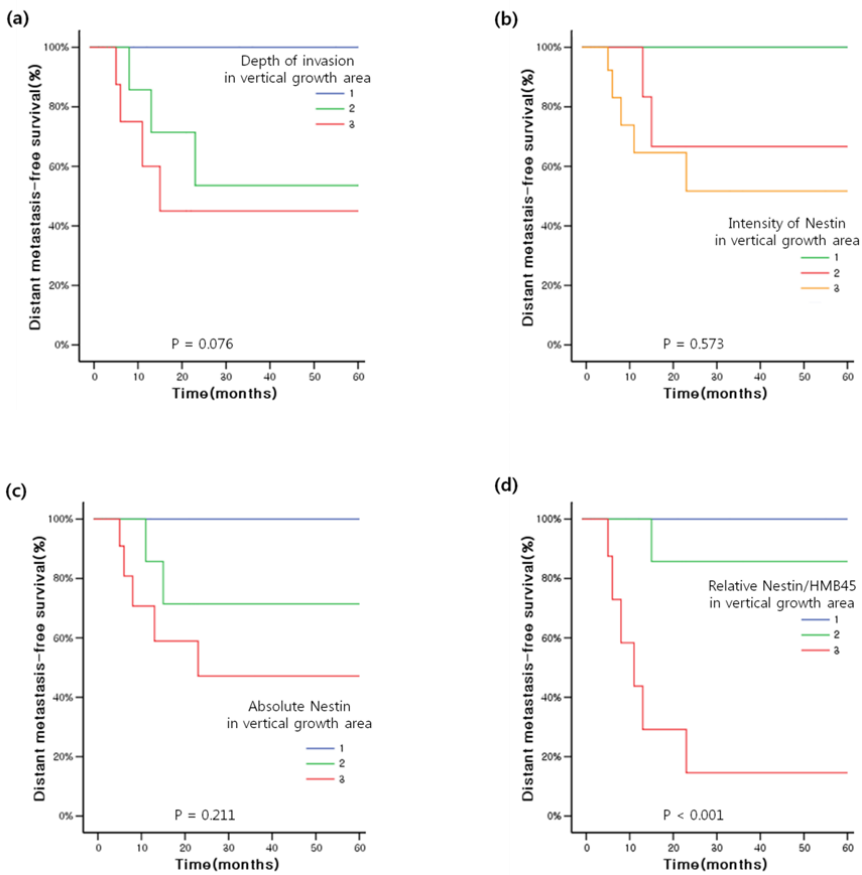


Figure 3. Kaplan-Meier curves for distant metastases of oral malignant melanoma according to according to (a) depth of invasion in the vertical growth area, (b) intensity of Nestin, (c) absolute staining level of Nestin, and (d) relative staining level of Nestin compared to HMB-45 (n=30).

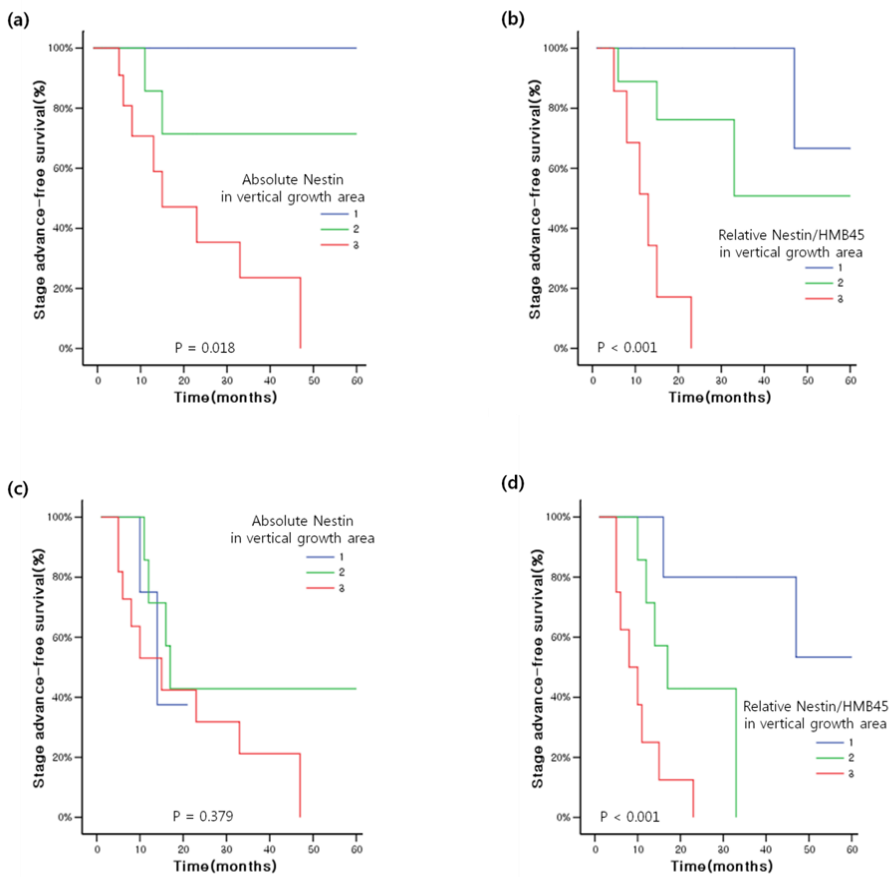


Figure 4. Kaplan-Meier curves for stage advance on the classifications of stage in mucosal melanoma of AJCC 2010 according to (a) absolute staining level of Nestin and (b) relative staining level of Nestin compared to HMB-45 in the vertical growth area. Kaplan-Meier curves for stage advance on the classifications of stage similar to cutaneous melanoma according to (c) absolute staining level of Nestin and (d) relative staining level of Nestin compared to HMB-45 in the vertical growth area (n=30).

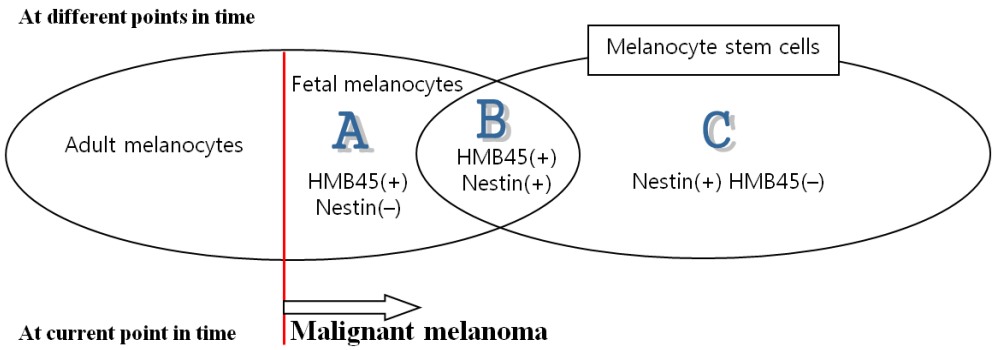


Figure 5. Hypothesis on the spatiotemporal expression of neuroepithelial stem cell marker Nestin and HMB-45. Adult melanocytes usually show no immunoreactivity for HMB-45 and Nestin, and fetal melanocytes (A area in figure) are immunoreactive for HMB-45 but not for Nestin. Melanocyte stem cells (C area in figure) have immunoreactivity for Nestin and show no reactivity for HMB-45. If we investigate human tissue at the current point in time, an increasing number of atypical melanocytes immunoreactive for HMB-45 are diagnostic of malignant melanoma. Our hypothesis is that malignant melanoma cells identified by immunoreactivity for HMB-45 and are more strongly reactive with an immunostain for Nestin depending on the level of dedifferentiation.