The Clinicopathological Significance of Epithelial Mesenchymal Transition Associated Protein Expression in Head and Neck Squamous Cell Carcinoma

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Background: Epithelial mesenchymal transition (EMT) has an important role in invasion and metastasis of tumor cells. The purpose of this study was to evaluate the roles of EMT-associated proteins on progression and metastasis as a prognostic/predictive factor in curatively-resected (R0) head and neck squamous cell carcinoma (HNSCC). **Methods:** A total of 118 patients who received curative surgery for HNSCC at Inha University Hospital between January 1996 and December 2011 were included. We used protein immunohistochemistry to evaluate the expression of E-cadherin, vimentin, and EZH2 on tissue microarrays. Also, we reviewed all medical records and analyzed the relationship between the expression of EMT-associated proteins and prognosis. **Results:** The E-cadherin–negative group showed more moderate/poor differentiation of cancer cell type than the higher E-cadherin–expressing group (p = .016) and high EZH2 expression was significantly correlated with nodal metastasis (p = .012). Our results demonstrate a significant association between high expression of EZH2 and vimentin and presence of distant progression (p = .026). However, expression of E-cadherin, vimentin, and EZH2 was not significantly associated with overall survival. **Conclusions:** These findings suggest that an EMT-associated protein expression profile is correlated with aggressiveness of disease and prognosis, and could be a useful marker for determination of additional treatment in curatively-resected HNSCC patients.

Squamous cell carcinoma is the most common malignant neoplasm of the head and neck. It was reported that there were 633,000 new registered cases and 355,000 deaths in 2008 worldwide.[1] There is a large geographic variability in the occurrence and the origin site of head and neck squamous cell carcinoma (HNSCC), which reflects the prevalence of tobacco and alcohol consumption, and ethnic and genetic differences among populations.

Although surgery has become standard practice and is endorsed by practice guidelines, recurrence remains a serious problem. We could optimize extended adjuvant radiotherapy and/or chemotherapy after surgery for patients with HNSCC if there are accurate biomarkers to predict prognosis. Also, we can achieve personalized cancer treatment in HNSCC patients by using these risk-stratified approaches. However, the prognostic or predictive biomarkers that can be used to predict which patient with curatively-resected HNSCC will develop a recurrence are very limited.

Epithelial-mesenchymal transition (EMT) means that the epithelial cells lose their properties of adhesion and polarization

and gain the properties of invasion and migration. Recently, EMT appeared to be a key factor in cancer metastasis, allowing tumor cells to leave the primary tumor environment to migrate as circulating tumor cells to distant sites.[2,3] E-Cadherin has an important role in the polarization of epithelial cells and its repression is the central target of EMT. In addition to aberrant expression of E-cadherin, vimentin has been associated with EMT.[3,4] Various types of tumors including HNSCC,[5-8] squamous cell carcinoma of the uterine cervix,[9] colorectal adenocarcinoma[10] and lung adenocarcinoma[11] have shown that the loss of E-cadherin expression or the expression of vimentin are significantly associated with poor prognostic factors such as lymph node metastasis, recurrence, overall survival and distant metastasis. The enhancer of zeste homolog 2 (EZH2) is an important molecule of the polycomb-repressive complex 2, which regulates gene expression.[12] Several reports have also shown that EZH2 is overexpressed in other aggressive tumors including lung cancer,[13] melanoma,[14] and bladder cancer.[15] In head and neck cancer, Wang *et al*.[5] showed that EZH2 over-expression was correlated with reduced overall survival in oral squamous cell carcinoma. Furthermore, over-expression of EZH2 was correlated with reduced expression of the tumor suppressor gene E-cadherin.[7] The other study also described a mechanism by which E-cadherin is repressed in EZH2-overexpressing cells through histone H3K27 trimethylation of the E-cadherin promoter.[8] These results suggest that EZH2 has an effect on EMT through regulation of E-cadherin expression. To clarify the exact role in HNSCC progression, we tried to examine the association between expression of these EMT-associated proteins and clinicopathological characteristics. Additionally, oropharyngeal squamous cell carcinoma is known to be related to infection with high-risk human papillomavirus (HPV), and a recent article[16] reported that the EZH2 gene was activated by an oncogene of HPV. Furthermore, they found that HPV-positive dysplastic lesions are characterized by a high level of EZH2 protein *in vivo*. We also analyzed the relationship of EMT-associated protein expression with HPV status, clinicopathologic features and overall survival.

MATERIALS AND METHODS

Patient selection

Patients who underwent curative surgery for HNSCC at Inha University Hospital between January 1996 and December 2011 were selected for this study. The primary sites of the tumors were the oral cavity, oropharynx, hypopharynx, and lar-

ynx. All patients received curative R0 resection. The patients' clinical and pathological characteristics regarding age, sex, smoking history, alcohol consumption, histologic types, pathologic TNM staging, relapse-free survival, and overall survival were obtained by a review of medical records. Thus, a total of 118 patients were eligible, according to the following criteria: histology of squamous cell carcinoma and the availability of hematoxylin and eosin-stained glass slides and paraffin blocks for construction of a tissue microarray (TMA). However, the smoking history of eight patients and the status of lymph node metastasis in one patient were not available. This study protocol was approved by the Ethics Committee (Institutional Review Board) of Inha University Hospital.

TMA and immunohistochemistry

We obtained formalin-fixed paraffin-embedded tissues of 118 patients for this study. The two representative areas of tumors were marked on glass slides. The criteria for defining the representative area were as follows: 1) invasive front of the tumor and 2) high percentage of tumor cells compared to surrounding stromal cells. To create TMAs, we punched two tissue columns (2.0 mm in diameter) from each original paraffin block and inserted them into the recipient paraffin blocks (each containing 30 to 69 holes). Six blocks of TMA were made for this immunohistochemical study.

Paraffin blocks of the TMA were sectioned at a 4-μm thickness. The sections were processed in an automated machine (BenchMarkXT, Ventana Medical Systems, Tucson, AZ, USA) for deparaffinization and then re-hydrated through graded alcohol. Epitope retrieval was performed by heating for 30 minutes and then incubating the slides for 32 minutes (37°C) with monoclonal antibody, followed by an incubation with a visualization reagent. Anti-E-cadherin (1:200, Zymed Laboratories, Inc., San Francisco, CA, USA), anti-vimentin (1:300, DAKO, Carpinteria, CA, USA), and anti-EZH2 (1:400, Novocastra, Bannockburn, IL, USA) were stained by the same method. Additionally, anti-p16 (BD Transduction Laboratories, BD Biosciences, mtm laboratories AG, Heidelberg, Germany) antibody was used to stain only the group with oropharynx cancer.

Analysis of immunohistochemical stains

For the evaluation of the expression of E-cadherin and vimentin, the proportion of positive tumor cells was visually estimated in two total cores. E-Cadherin with membranous staining was classified into three categories[6]: 1) strong (S) pattern: almost all tumor cells showed diffuse patterns and strong positive

staining in the membrane; 2) weak and homogeneous (W&H) pattern: tumor cells were uniformly, but more weakly stained; 3) heterogeneous (HEG) pattern: tumor cells showed focal staining with variable intensity. W&H and HEG patterns were considered to represent a loss of E-cadherin expression.

Vimentin expression was interpreted as positive when cytoplasmic staining was observed, even in a small portion of the tumor cells (at least 5%) at the invasive front. However, the tumor cells in the basal layer were excluded in the interpretation, because they were frequently positive for vimentin in almost all cases. Diffuse expression of vimentin was also regarded as positive. The intensity of staining was not considered in the evaluation.

The nuclear staining of EZH2 was evaluated semi-quantitatively on the basis of staining intensity and distribution using the immunoreactive score[13,14,17]: immunoreactive score = intensity score × proportion score. The intensity score was defined as follows—0, negative; 1, weak; 2, moderate; or 3, strong, and the proportion score was defined as 0, negative; 1, < 10%; 2, 11–50%; 3, 51–80%; 4, > 80% positive cells. The proportion of the immunoreactive tumor cells was estimated in one high-power field (× 400) of the hot spot. The total score ranged from 0 to 12. Low expression of EZH2 was defined as a total score of 0 to 4, and high expression was defined as a total score > 4. The p16-positive cases showed diffuse and strong nuclear expression in all cases, and the other cases were negative for p16 without any ambiguous cases.

Statistical analysis

A Pearson's chi-squared test and independent-sample t-test were used to determine the statistical significance of differences between the positive and negative immunoreactive groups for E-cadherin, vimentin, and EZH2 of HNSCC in terms of sex, age, smoking history, primary tumor site, histological differentiation, tumor stage, resection margin status, node metastasis, recurrence, and survival rate. The Kaplan-Meier method was used for survival analysis. The overall survival was determined by measuring the time interval from the beginning of the treatment to the date of death. We censored the patients who were alive or were lost during the follow-up in the data analysis. All statistical analyses were conducted using statistical software PASW Statistics ver. 18.0 (SPSS Inc., Chicago, IL, USA) and p-values less than .05 were considered statistically significant.

RESULTS

We analyzed 118 patients (101 men [85.6%] and 17 women [14.4%]), with a median age of 58 years (range, 27 to 94 years). The tumors were located in the oral cavity (33.9%, 40 cases), oropharynx (18.6%, 22 cases), hypopharynx (19.5%, 23 cases), and larynx (28.0%, 33 cases). Eighty-six cases (72.9%) exhibited strong and homogenous membranous E-cadherin expression. The loss of E-cadherin was found in 32 cases (27.1%) (Fig. 1A–D). The expression of vimentin was frequently observed in tumor cells of the invasive front, especially abutting adjacent stroma. Twenty-nine cases (24.6%) exhibited focal or diffuse cytoplasmic immmunoreactivity for vimentin (Fig. 1E–H). According to the immunoreactive score, high expression of EZH2 was observed in 29 cases (24.6%) (Fig. 1I–L). Expression of p16 was observed in 14 of 22 cases (63.6%) of oropharyngeal cancer.

Association of the clinicopathological parameters with the expression levels of E-cadherin, vimentin, and EZH2

The results of immunohistochemical staining and its association with clinicopathological parameters are summarized in Table 1. The E-cadherin–negative group showed more moderately/poorly differentiated cell types than the higher E-cadherin–expressing group (62.8% vs 87.5%, p = .016). High EZH2 expression was significantly correlated with nodal metastasis (p = .012). In the subgroup composed of only oral cavity tumors, the expression of vimentin was associated with a higher tumor stage (T stage [vimentin negative/positive]; T1 (10/5), T2 (14/5), T3 (2/0), T4 (0/4), p = .027).

Association of the overall survival with the expression levels of E-cadherin, vimentin, and EZH2

The association between vimentin, E-cadherin, EZH2 expression and clinicohistologic parameters with survival rate was evaluated by Cox proportional hazard model. Comparing the clinicohistologic parameters, pathologic tumor stage (odds ratio, 2.541; p < .001), pathologic nodal stage (odds ratio, 2.043; p = .009), TMN stage (odds ratio, 2.233; p = .006), extracapsular extension (odds ratio, 1.982; p = .045), and margin status (odds ratio, 2.956; p < .001) were shown to be significantly associated with survival rate. No significant differences were found for sex, alcohol consumption, smoking history, and histologic differentiation of tumors. Furthermore, the expression of E-cadherin, vimentin, and EZH2 was not significantly associated with overall survival. However, in an analysis according to primary tumor site subgroups, the loss of E-cadherin was associated with lower overall survival in oropharyngeal and hypopharyngeal tumors (p = .001 and p = .038, respectively) (Fig. 2).
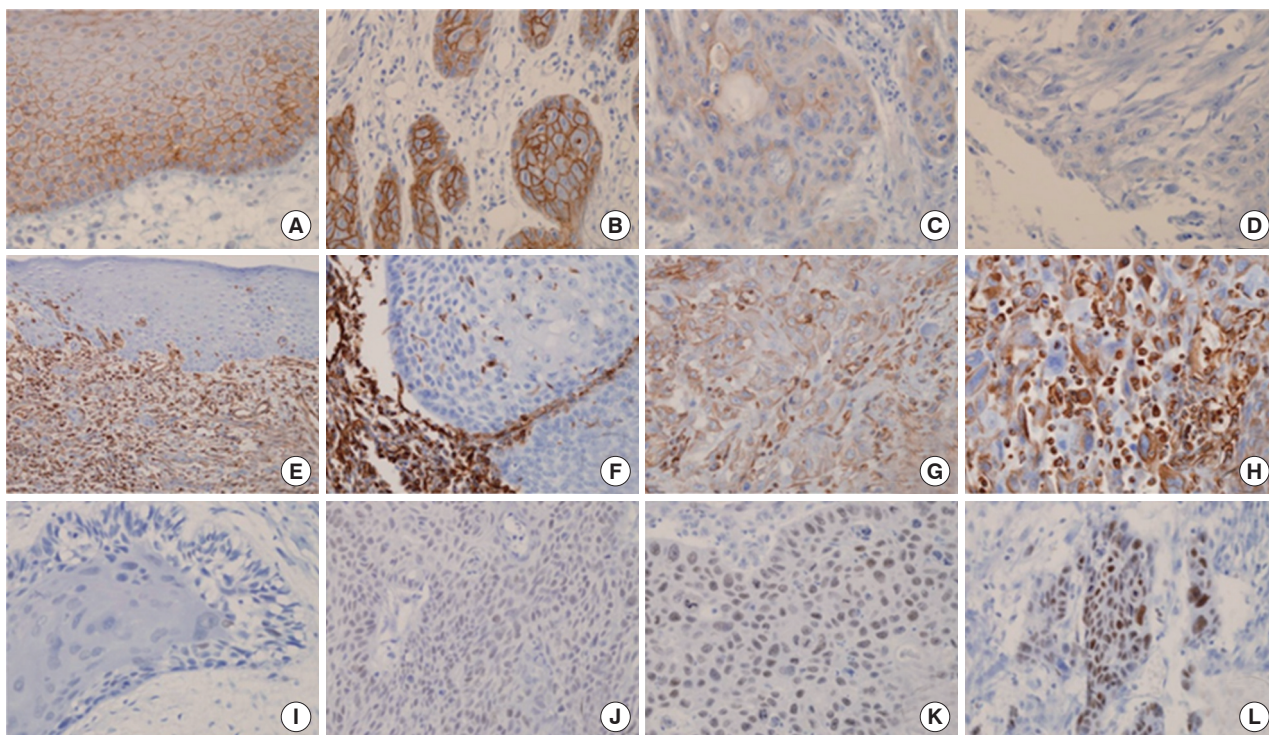
Fig. 1. The expression of E-cadherin (A–D), vimentin (E–H), and EZH2 (I–L) in normal squamous epithelium and tumors. E-Cadherin is expressed in the membranes of normal cells (A) and tumor cells (B), but some tumors show heterogeneous (C), or loss (D) of expression. Normal squamous epithelium (E) and squamous cell carcinoma (F) are negative for vimentin, but a portion of tumors show intermediate (G) or strong (H) expression of vimentin in the cytoplasm of tumor cells. Weak and focal (I) or weak and diffuse (J) expression of EZH2 is grouped as low immunoreactivity. Moderate and diffuse (K) or strong and diffuse (L) expression of EZH2 is grouped as high immunoreactivity.

The expression of p16 and EMT-associated protein in oropharyngeal cancer

In the oropharynx tumor group, the recurrence rate was significantly higher than that in the E-cadherin–negative group (loss, 2/4 [50%]; E-cadherin–expressing group, 1/18 [5.6%]; $p = .019$). Twenty-nine cases of all 118 HNSCC showed overexpression of EZH2, especially in the oropharynx tumor group (41.4%, $p = .002$). HPV infection is a well-known biomarker in oropharyngeal squamous cell carcinoma. In our study, expression of p16, a well-known surrogate marker in oropharyngeal cancer, was found in 14 of 22 cases of oropharynx cancer. In these patients, the rate of EZH2 expression according to p16 status was not statistically different ($p = .225$). However there was no recurrence in any of the p16-positive cases. In contrast, three of eight cases (37.5%) showed recurrence in the p16-negative group. The difference in the recurrence ratio was statically significant ($p = .014$). Oropharyngeal squamous cell carcinoma patients who had a smoking history showed more frequent, but not statistically significant differences in, EZH2 expression compared patients who had never smoked (72.7% vs 36.3%, $p = .094$).

DISCUSSION

In this study, we found that the EMT-associated protein expression profile was a strong prognostic marker for the entire HNSCC spectrum. Loss of E-cadherin expression is significantly associated with recurrence rate in oropharyngeal tumors, as well as overall survival in oropharyngeal and hypopharyngeal tumors. EZH2 and/or vimentin expression is significantly associated with more distant metastasis. Our study also suggests that this protein expression profile analysis may be helpful to identify patients at high risk of developing distant metastasis in early stage node-negative HNSCC patients.

E-Cadherin is a key molecule involved in the maintenance of intracellular adhesion, and down-regulation of E-cadherin is associated with tumor progression in diverse human cancer types.[18] There have been several reports regarding the inverse correlation between EZH2 and E-cadherin expression in cancer cells. However, the exact underlying mechanism by which EZH2 causes a poor prognosis is not known and further analyses are necessary to elucidate how EZH2 regulates E-cadherin expression. In our study, each of the factors was found to be associated with a pat-

Table 1. Relationship between EMT-associated protein expression and clinicopathological parameters

Characteristic	All patients	E-Cadherin positive	E-Cadherin loss	p-value	Vimentin negative	Vimentin positive	p-value	EZH2 negative	EZH2 positive	p-value
Sex										
Male	101	76	25	.159	79	22	.086	74	27	.185
Female	17	10	7		10	7		15	2	
Age (yr)	58.0±11.9	59.0±11.7	55.3±12.3	.520	59.0±11.7	55.0±12.2	.637	57.3±11.6	60.1±12.8	.811
Smoking history										
Never smoker	32	22	10	.647	24	8	.943	25	7	.494
Smoker or ex-smoker	78	57	21		59	19		56	22	
Unknown	8									
Pirmary tumor site										
Oral cavity	40	28	12	.564	26	14	.159	36	4	.002
Oropharynx	22	18	4		17	5		10	12	
Hypopharynx	23	18	5		17	6		18	5	
Larynx	33	22	11		29	4		25	8	
Histological differentiation										
Well	36	32	4	.016	27	9	.944	29	7	.391
Moderately/pooly	82	54	28		62	20		60	22	
Pathological tumor stage										
T1	30	20	10	.167	21	9	.135	20	10	.342
T2	51	42	9		42	9		38	13	
T3	18	13	5		15	3		14	4	
T4	19	11	8		11	8		17	2	
Lymph node invovement										
Absence	60	40	20	.136	46	14	.709	51	9	.012
Present	57	45	12		42	15		37	20	
Unknown	1									
Recurrence										
Free	89	66	23	.585	67	22	.950	69	20	.352
Present	29	20	9		22	7		20	9	
Follow-up										
Live	70	55	15	.093	54	16	.600	51	19	.434
Died	48	31	17		35	13		38	10	
Follow-up (mo)	46.2±38.7	45.4±37.4	48.3±42.7	.639	48.1±39.4	40.3±36.6	.571	50.0±41.5	34.6±25.6	.003

Values are presented an number or mean±standard deviation.

tern of metastasis and prognosis. However, we could not confirm a significant correlation between EZH2/vimentin expression and the recurrence rate or overall survival. There was a trend of a lower distant metastasis rate in patients with lower EZH2 expression compared to patients with high E-cadherin expression. However, this finding was not statistically significant (p=.192). The significance of E-cadherin expression as a predictive factor in metastatic spread is not clear.

There is *in vivo* and *in vitro* data demonstrating that EZH2 plays a crucial role in several steps of the metastatic process and that it is activated in endothelial cells in response to pro-angiogenic signals.[19] In our study, we found that there was a statistically significant difference in metastatic pattern according to mesenchymal markers expression. Given these hypotheses, our results suggest that mesenchymal markers could be very important biomarkers of distant metastasis in HNSCC.

Thus far, there is no conclusive proof that the markers related

to progression are directly correlated with prognosis in HNSCC. A recent TMA study of E-cadherin expression in oropharyngeal squamous cell carcinoma[20] failed to show a significant correlation between the expression of E-cadherin and histologic type, nodal and distant metastasis, suggesting that E-cadherin expression may not be a predictor of nodal or distant metastasis in these tumors. The inconsistent results in several studies are limit the use of these markers to predict patient outcome. We think that the differences in methods for analyzing immunohistochemistry may be an important factor affecting the results.

There are some limitations to our findings. Our analysis included all types of HNSCC and none of the subtypes had enough cases. Furthermore, our analysis was a single-center retrospective study. A large cohort study is necessary to confirm the significance of these markers.

In conclusion, EMT-associated protein expression is related to aggressive pathological features, even in early stage HNSCC.
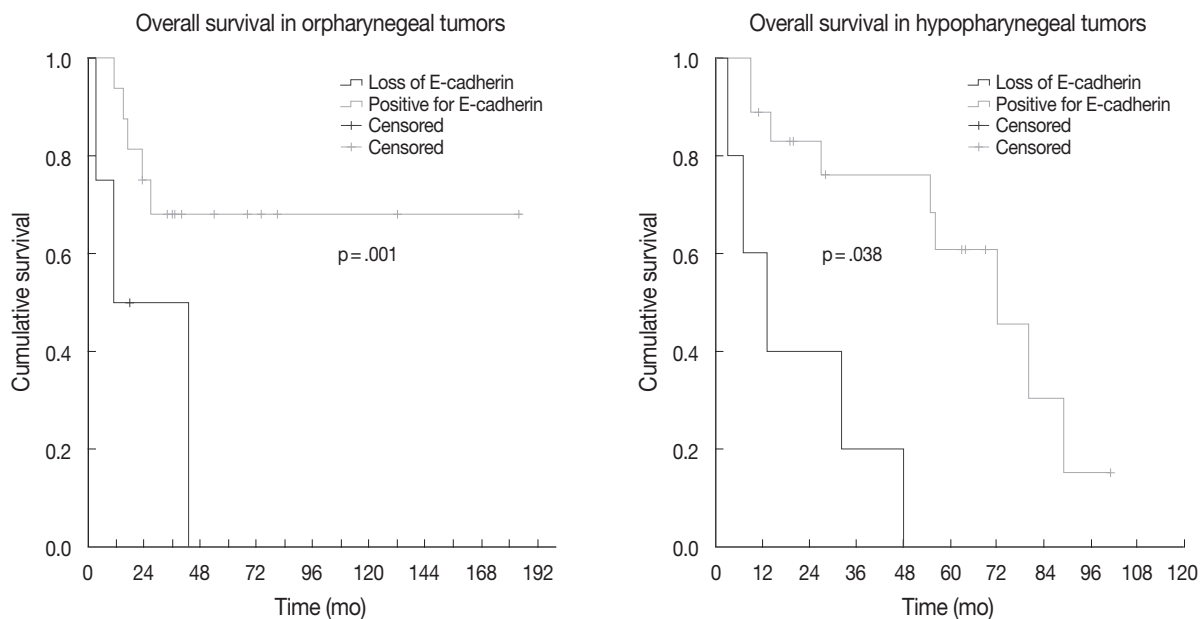
Fig. 2. The effects of E-cadherin on prognosis. Kaplan-Meier plots of overall survival in oropharyngeal and hypopharyngeal groups defined by expression of E-cadherin. The loss of E-cadherin is significantly correlated with the lower overall survival (p = .001 and p = .038, respectively).

Therefore, EMT-associated proteins could be useful markers for determination of additional treatment (e.g., adjuvant chemotherapy and/or radiotherapy) in curatively-resected HNSCC patients in the future.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-917.

2. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009; 139: 871-90.

3. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2: 442-54.

4. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006; 7: 131-42.

5. Wang C, Liu X, Chen Z, *et al*. Polycomb group protein EZH2-mediated E-cadherin repression promotes metastasis of oral tongue squamous cell carcinoma. Mol Carcinog 2013; 52: 229-36.

6. Nijkamp MM, Span PN, Hoogsteen IJ, van der Kogel AJ, Kaanders JH, Bussink J. Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients. Radiother Oncol 2011; 99: 344-8.

7. Ueda G, Sunakawa H, Nakamori K, *et al*. Aberrant expression of beta- and gamma-catenin is an independent prognostic marker in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2006; 35: 356-61.

8. Pyo SW, Hashimoto M, Kim YS, *et al*. Expression of E-cadherin, P-cadherin and N-cadherin in oral squamous cell carcinoma: correlation with the clinicopathologic features and patient outcome. J Craniomaxillofac Surg 2007; 35: 1-9.

9. Myong NH. Loss of E-cadherin and acquisition of vimentin in epithelial-mesenchymal transition are noble indicators of uterine cervix cancer progression. Korean J Pathol 2012; 46: 341-8.

10. Hong R, Choi DY, Lim SC, Suh CH, Kee KH, Lee MJ. The differential expressions of the epithelial-mesenchymal transition regulator, slug and the cell adhesion molecule, E-cadherin in colorectal adenocarcinoma. Korean J Pathol 2008; 42: 351-7.

11. Kim H, Yoo SB, Sun P, *et al*. Alteration of the E-cadherin/beta-catenin complex is an independent poor prognostic factor in lung adenocarcinoma. Korean J Pathol 2013; 47: 44-51.

12. Tan J, Yang X, Zhuang L, *et al*. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively

induces apoptosis in cancer cells. Genes Dev 2007; 21: 1050-63.

13. Breuer RH, Snijders PJ, Smit EF, *et al*. Increased expression of the EZH2 polycomb group gene in BMI-1-positive neoplastic cells during bronchial carcinogenesis. Neoplasia 2004; 6: 736-43.

14. Bachmann IM, Halvorsen OJ, Collett K, *et al*. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 2006; 24: 268-73.

15. Weikert S, Christoph F, Köllermann J, *et al*. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int J Mol Med 2005; 16: 349-53.

16. Holland D, Hoppe-Seyler K, Schuller B, *et al*. Activation of the enhancer of zeste homologue 2 gene by the human papillomavirus E7 oncoprotein. Cancer Res 2008; 68: 9964-72.

17. Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL, Jiang HB. Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. Mod Pathol 2010; 23: 213-24.

18. Diniz-Freitas M, García-Caballero T, Antúnez-López J, Gándara-Rey JM, García-García A. Reduced E-cadherin expression is an indicator of unfavourable prognosis in oral squamous cell carcinoma. Oral Oncol 2006; 42: 190-200.

19. Liu M, Scanlon CS, Banerjee R, *et al*. The histone methyltransferase EZH2 mediates tumor progression on the chick chorioallantoic membrane assay, a novel model of head and neck squamous cell carcinoma. Transl Oncol 2013; 6: 273-81.

20. Ukpo OC, Thorstad WL, Zhang Q, Lewis JS Jr. Lack of association of cadherin expression and histopathologic type, metastasis, or patient outcome in oropharyngeal squamous cell carcinoma: a tissue microarray study. Head Neck Pathol 2012; 6: 38-47.