Original Article

Immunohistochemical analysis of tumor budding as predictor of lymph node metastasis from superficial esophageal squamous cell carcinoma

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Abstract

Background Tumor budding is known predictors of lymph node metastasis from esophageal squamous cell carcinoma. However, it is not easy to detect such small cell clusters on hematoxylin-eosin (HE) staining. Therefore, we evaluated tumor budding using immunohistochemistry (IHC) for epithelial cell markers.

Method We analyzed tumor budding in 50 cases of superficial esophageal squamous cell carcinoma. We evaluated the impact of clinicopathological factors and tumor budding to predict lymph node metastasis. A total of 565 tumor sections were assessed by using HE staining and IHC for cytokeratin 5/6.

Results Based on receiver operating characteristic curves, the cut-off values for high-grade tumor budding evaluated by using HE staining or IHC were 2 and 11, respectively. High-grade tumor budding evaluated by using HE staining (P=0.007) and IHC (P≤0.001) were significantly correlated with lymph node metastasis. For tumors with pT1a-MM to pT1b-SM1, high-grade tumor budding evaluated by using IHC was correlated with lymph node metastasis (P=0.050).

Conclusions Tumor budding was significantly associated with lymph node metastasis. The optimal cut-off values of tumor budding on HE staining and tumor budding on IHC were 2 and 11, respectively. Even though both tumor budding on HE staining and tumor budding on IHC were significantly associated with lymph node metastasis, tumor budding on IHC tend to be more associated with lymph node metastasis.

Keywords

tumor budding, immunohistochemistry, esophageal squamous cell carcinoma, lymph node metastasis

Abbreviations

SCC	squamous cell carcinoma
SM	submucosal layer
MM	muscularis mucosae
LPM	lamina propria mucosae
HE	hematoxylin-eosin
IHC	immunohistochemistry

Introduction

Lymph node metastasis is associated with tumor depth and/or lymphovascular invasion in superficial esophageal squamous cell carcinoma (SCC). Lymph node metastasis is not observed from tumors in the epithelium (EP) and is rare from tumors in the lamina propria mucosae (LPM) [1, 2]. Some tumors in the muscularis mucosae (MM) and in the upper third of the submucosal layer (SM1) may result in lymph node metastasis [1,2]. In these subgroups, some cases show lymph node metastasis without lymphovascular invasion; therefore, other parameters should be developed to predict lymph node metastases.

Tumor budding was reported to be histopathological predictor of lymph node metastasis or poor prognosis in gastrointestinal carcinoma including esophageal squamous cell carcinoma [3-6]. Tumor budding is defined as a cancer cell nest consisting of one or fewer than five cells that infiltrates the interstitium at the invasive margin of the cancer [4]. Cases with 5 or more tumor budding in a single field observed through a 20x objective lens are classified as high risk of lymph node metastasis.

However, hematoxylin-eosin (HE) staining is not enough to detect such small cell clusters. Takamatsu et al. reported the usefulness of immunohistochemistry (IHC) to detect tumor budding by using epithelial cell markers to predict lymph node metastasis from T1 colorectal cancer [8]. To the best of our knowledge, no studies have evaluated the correlation between tumor budding evaluated by using IHC for epithelial cell markers and lymph node metastasis from esophageal superficial SCC.

Therefore, we evaluated the clinical impact of tumor budding to predict lymph node metastasis from superficial esophageal SCC by using HE staining and IHC.

Methods

Patients

We retrospectively analyzed 565 sections. The samples were obtained from 50 patients with superficial esophageal SCC, 40 men and 10 women with a median age of 67 (range; 49–85) years. The patients underwent radical surgery without neoadjuvant therapy at Toho University Hospital (Tokyo, Japan) between 2004 and 2018. Two patients underwent endoscopic resection before surgery.

All 33 pT1b-SM tumor specimens were classified into two groups according to the Japanese Classification of Esophageal Cancer: tumors invading up to 200 μm from the MM were classified as pT1b-SM1 and tumors invading more than 200 μm were classified as pT1b-SM2 [9]. We examined the correlations between lymph node metastasis and clinicopathological variables such as age, gender, longitudinal diameter, tumor depth, lymphovascular invasion, and tumor budding. According to the tumor depth, there was 1 case of pT1a-EP, 5 of pT1a-LPM, 11 of pT1a-MM, 5 of pT1b-SM1, and 28 of pT1b-SM2. A total of 9 cases had lymph node metastasis.

Histological examination

All resected specimens were fixed with 10% buffered formalin; then, the surgical resection specimens

were cut into 5-mm to 6-mm thick sections, and the endoscopic resection specimens were cut into 2-mm to 3- mm thick sections according to the Japanese Classification of Esophageal Cancer [9]. The paraffin-embedded tissue sections were sliced at a thickness of 3 μm. Each section was stained by using HE. We used D2-40- stained sections (D2-40, dilution 1:2, Nichirei Biosciences, Tokyo, Japan) and Elastica van Gieson-stained sections to detect lymphatic invasion and venous invasion, respectively.

We evaluated tumor budding by referring to the definitions and methods reported by Ueno et al [10]. Tumor budding was defined as a cancer cell nest consisting of one or fewer than five cells that infiltrates the interstitium at the invasive margin of the cancer. First, we selected the site where tumor budding was most frequent at the invasive frontal region. Second, we counted the number of tumor buddings in a single field measuring 0.95 mm^2 by using the 10× eyepiece with field number 22 and 20× objective lens. We evaluated tumor budding by using HE-stained sections and cytokeratin 5/6-immunostained sections (D5/16 B4, dilution 1:100, DAKO, Glostrup, Denmark; Fig. 1). Cytokeratin 5/6 is a subtype of cytokeratin expressed by squamous cells.

The IHC procedures were performed by using an auto-stainer. IHC for D2-40 and cytokeratin 5/6 was performed using a BenchMark ULTRA and a BenchMark XT (Ventana Medical Systems, Tucson, USA), respectively. We performed staining according to the procedures specified by the manufacturing company. Tumor budding were evaluated by the pathologist in the absence of clinicopathological information including lymph node metastasis.

Statistical analysis

We assessed the correlations between clinicopathological variables and lymph node metastasis using the Mann-Whitney *U* test or Fisher's exact probability test. We determined cut-off values for tumor budding by using receiver operating characteristic curves. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). The EZR is a modified version of R commander designed to add statistical functions frequently used in biostatistics [11].

Results

Correlations between clinicopathological factors and lymph node metastasis

Correlations between various risk factors and lymph node metastasis are shown in Table 1. Tumor size (P=0.062), tumor depth (P=0.107) and lymphatic invasion (P=0.140) was slightly associated with lymph node metastasis. Lymphatic invasion was observed in 21 of 50 patients (42.0%), and was detected in 6 of 9 patients (66.7%) including lymph node metastasis.

The accuracy of lymphovascular invasion, lymphatic invasion, and venous invasion for predicting lymph node metastases is shown in Table 2. Although the sensitivity of lymphovascular invasion and lymphatic invasion were both 66.7%, the specificity of lymphatic invasion (63.4%) was higher than that of lymphovascular invasion (36.6%).

Tumor budding

Figure 2 shows the number of tumor buddings evaluated by using HE staining and IHC for each case, as well as the receiver operating characteristic curves for each staining method. On HE staining, the mean number of tumor budding was 1 (range, 0–25) in cases without lymph node metastasis and 6 (range, 1–12) in cases with lymph node metastasis (Fig. 2a). Based on receiver operating characteristic curves used to predict lymph node metastases, the cut-off value for high-grade tumor budding was 2 (Fig. 2b). On IHC, the mean number of tumor budding was 3 (range, 0–46) in cases without lymph node metastasis and 14 (range, 11–35) in cases with lymph node metastasis (Fig. 2c). Based on receiver operating characteristic curves, the cut-off value for high-grade tumor budding was 11 (Fig. 2d).

The accuracy of tumor budding using HE staining and IHC to predict lymph node metastases is shown in Table 3. On HE staining, the sensitivity and specificity of the cases with high-grade tumor budding (≥2 tumor budding) were 88.9% and 65.9%, respectively. High-grade tumor budding was significantly associated with lymph node metastasis (P=0.007). Based on IHC, the sensitivity and specificity of the cases with high-grade tumor budding (≥11 tumor budding) were 100% and 73.2%, respectively. High-grade tumor budding was significantly associated with lymph node metastasis (P<0.001).

The relationships between lymph node metastasis and high-grade tumor budding evaluated by HE staining and IHC are summarized in Figure 3. High-grade tumor budding evaluated by using HE staining showed slightly more false-positive cases than that evaluated by using IHC. Although high-grade tumor

budding evaluated by using HE staining showed 1 false-negative case, high-grade tumor budding evaluated by using IHC did not reveal any false-negative cases. Among high-grade tumor budding evaluated by using HE staining and high-grade tumor budding evaluated by using IHC, high-grade tumor budding evaluated by using IHC tended to be more associated with lymph node metastasis.

Relationships between pathological findings and lymph node metastasis in cases of pT1a-MM to pT1b-SM1

Table 4 shows the relationships between clinicopathological findings and lymph node metastasis in cases of pT1a-MM to pT1b-SM1. Both the patients with lymph node metastasis were positive for high-grade tumor budding evaluated by using HE staining and IHC. In contrast, both patients were negative for lymphovascular invasion. The sensitivity and specificity of high-grade tumor budding evaluated by using IHC were 100% and 85.7%, respectively. Although the difference was not significant, high-grade tumor budding evaluated by using IHC was associated with lymph node metastasis (P=0.050).

Discussion

Tumor budding was significantly associated with lymph node metastasis from esophageal superficial SCC. The optimal cut-off value for tumor budding on HE staining was 2, and that for tumor budding on IHC was 11. Both the sensitivity and specificity for tumor budding on IHC were better than those for tumor budding on HE staining were.

The results of our study supported those of previous studies about the usefulness of tumor budding to predict lymph node metastasis [7, 12]. On the other hand, tumor depth or lymphatic invasion reported as useful predictors did not show significant correlation with lymph node metastasis in this present study. All the tumors were pT1 which underwent radical surgery without neoadjuvant therapy. Therefore, there were few pT1a tumors. As a result, there was a difference in the distribution of patients for each tumor depth. In addition, three of 9 patients including lymph node metastasis were negative for lymphatic invasion. These are considered to be the reason why tumor depth or lymphatic invasion did not show a significant correlation with lymph node metastasis in this present series.

Cancer cell clusters that detach from the primary tumor tissue are likely to show stromal invasion of carcinoma. Therefore, tumor budding may be indicator of stromal invasion. Carcinomas develop lymph node metastasis thorough stromal invasion and lymphatic invasion. Therefore, tumor budding may be the cause of lymph node metastasis. In fact, in the present study, all cases with lymph node metastasis showed at least one tumor budding in one high power field.

Tumor budding on HE staining and tumor budding on IHC were more accurate than lymphovascular invasion. Therefore, these may be appropriate predictors for lymph node metastasis. Regarding the cut-off value for the number of small cancer cell clusters, 2 was decided to be the optimal cut-off value of high-grade tumor budding on HE staining. This cut-off value was very close to the cut-off value of 3 reported by Teramoto et al. for superficial esophageal SCC [12]. In the present study, the optimal cut-off

value for high-grade tumor budding on IHC was higher than that tumor budding on HE staining, similar to the finding in a previous study using IHC for T1 colorectal cancer [8]. The difference in the optimal cut-off value between HE staining and IHC might be owing to the difficulty in counting tumor budding on HE staining. Moreover, HE staining might not be enough to differentiate between fibroblast proliferation and/or inflammatory cell infiltration from cancer cells at the invasive frontal region [10]. The small number of patients we examined in this study was a limitation. Multivariate analysis should be performed to evaluate tumor budding as an independent predictor of lymph node metastasis. However, the number of patients, especially those with lymph node metastasis, were not sufficient to perform the multivariate analysis. Diagnostic test statistics should also be performed in another cohort to confirm the usefulness of cut-off value obtained in this study. However, we could not confirm in another cohort in this study because of the limited number of patients with T1. In fact, in clinical settings, most patients with pT1a-MM or pT1b-SM1 esophageal SCC are treated with chemoradiotherapy or endoscopic resection. Although limited numbers of patients with these tumors are treated with surgery, further research is needed to evaluate the predictive impact of tumor budding evaluated by using IHC. The other limitation was the thickness of the 5-6mm slides in our study. This thickness might be a cause of false negative to detect lymphovascular invasion.

In conclusion, tumor budding was significantly associated with lymph node metastasis. Based on receiver operating characteristic curves, the optimal cut-off values of tumor budding on HE staining and

tumor budding on IHC were 2 and 11, respectively. Even though both tumor budding on HE staining and tumor budding on IHC were significantly associated with lymph node metastasis, tumor budding on IHC tend to be more associated with lymph node metastasis. However, the number of patients in this present study were not enough to make rigid conclusion.

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Compliance with ethical standards

Ethical statement: This study was approved by the Toho University Medical Center Omori Hospital Ethics Committee (Approval number: M18015). This retrospective observational study was performed by opt-out method on the web site of the Toho University Omori Medical Center.

Conflict of interest: Dr. Shimada reports grants from TAIHO Pharmaceutical, Co. ltd., grants from Chugai Pharmaceutical, Co. ltd., personal fees from M3/CIWORKS, personal fees from ONO PHARMACEUTICAL CO., LTD., personal fees from Takeda Pharmaceutical Company Limited., grants from Eli Lilly Japan K.K, personal fees from DAIICHI SANKYO HEALTHCARE CO.,LTD., grants and personal fees from Yakult Honsha Co., Ltd., grants from Roche Diagnostics K.K., personal fees from

Nippon Kayaku Co, Ltd, personal fees from Oncolys BioPharma Inc., personal fees from EA Pharma Co., Ltd., outside the submitted work; . The other authors have no conflict of interest to declare.

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Figure Legends

Fig. 1. Histopathological findings of tumor budding (arrows) in T1 esophageal squamous cell carcinoma on (a) HE staining and (b) cytokeratin5/6 IHC.

Fig. 2. Number of tumor budding for each case evaluated by HE staining (a), and cytokeratin 5/6 IHC (c). Receiver operating characteristic curve of tumor budding evaluated by HE staining (b), and cytokeratin 5/6 IHC (d).

Fig. 3. Relationships between lymph node metastasis, high-grade tumor budding evaluated by HE staining and high-grade tumor budding evaluated by IHC in cases of pT1a-EP to pT1b-SM2.

Table 1. Clinical characters of the patients.

Risk factors	Lymph node metastasis		*P* value
	Positive(n=9)	Negative(n=41)	
Age(year)			
Median	67(61-79)	66(49-85)	
>67	4	20	1.000
=67	5	21	
Gender			
Male	8	32	0.665
Female	1	9	
Tumor size(mm)			
Median	42 (25-80)	25(9-170)	
>30	7	16	0.062
=30	2	25	
Tumor depth			
T1a-EP	0	1	0.107
T1a-LPM	0	5	
T1a-MM	1	10	
T1b-SM1	1	4	
T1b-SM2	7	21	
Lymphovascular invasion			
Positive	6	26	1.000
Negative	3	15	
Lymphatic invasion			
Positive	6	15	0.140
Negative	3	26	
Venous invasion			
Positive	4	19	1.000
Negative	5	22	

Table 2. Accuracy of vessel permeation

	sensitivity (%)	specificity (%)	PPV (%)	NPV (%)	*P* value
Lymphovascular invasion	66.7	36.6	18.8	83.3	1.000
Lymphatic invasion	66.7	63.4	28.6	89.7	0.140
Venous invasion	44.4	53.7	17.4	81.5	1.000

PPV: Positive predictive value, NPV: Negative predictive value

Table 3. Accuracy of tumor budding

Tumor budding	Lymph node metastasis		sensitivity (%)	specificity (%)	PPV (%)	NPV (%)	*P* value
	positive	negative					
HE staining							
High grade (=2)	8	14	88.9	65.9	36.4	96.4	0.007
Low grade (<2)	1	27					
Immunohistochemistry							
High grade (=11)	9	11	100	73.2	45.0	100	<0.001
Low grade (<11)	0	30					

PPV : Positive predictive value, NPV : Negative predictive value

Table 4. Relationships between lymph node metastasis and pathological parameters (pT1a-MM to pT1b-SM1)

	Lymph node metastasis						
	positive	negative	sensitivity (%)	specificity (%)	PPV (%)	NPV (%)	*P* value
Tumor budding							
HE staininig							
High grade (≥2)	2	5	100	64.3	28.6	100	0.175
Low grade (<2)	0	9					
Immunohistochemistry							
High grade (≥11)	2	2	100	85.7	50.0	100	0.050
Low grade (<11)	0	12					
Lymphovascular invasion							
Positive	0	9	0	35.7	0	71.4	0.175
Negative	2	5					
Lymphatic invasion							
Positive	0	7	0	50.0	0	77.8	0.475
Negative	2	7					
Venous invasion							
Positive	0	5	0	64.3	0	81.8	1.000
Negative	2	9					

PPV : Positive predictive value, NPV : Negative predictive value

Fig. 1

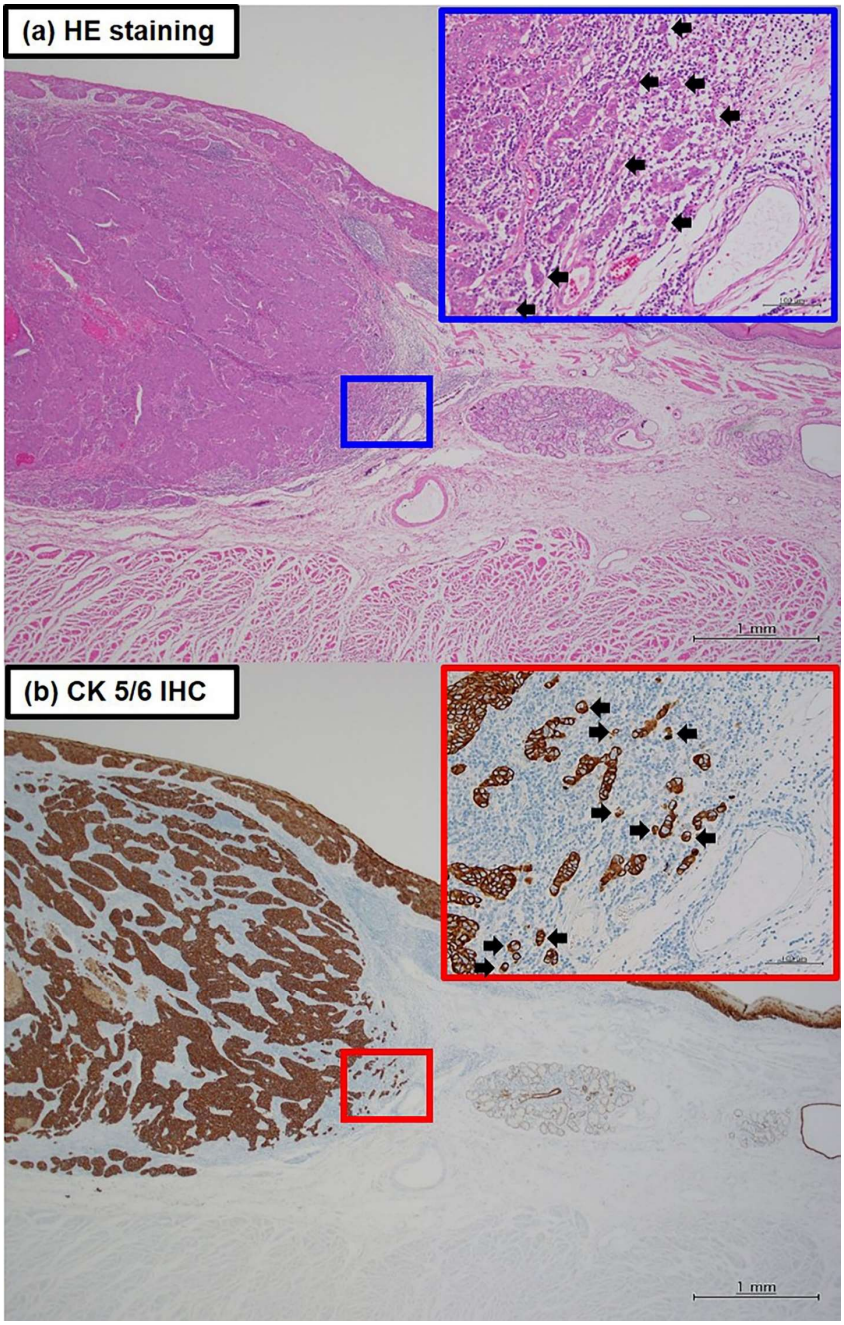
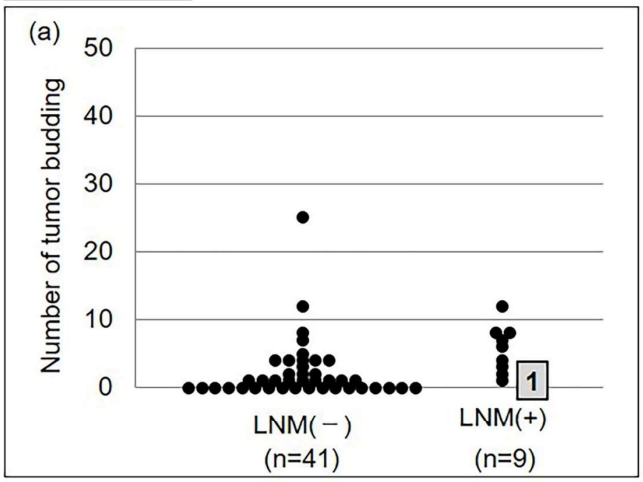
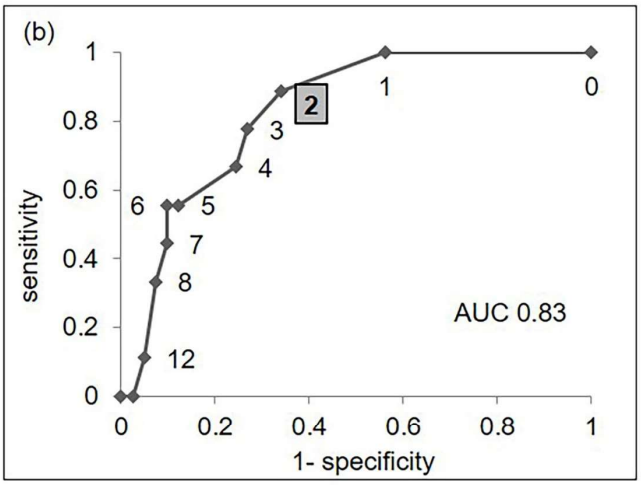


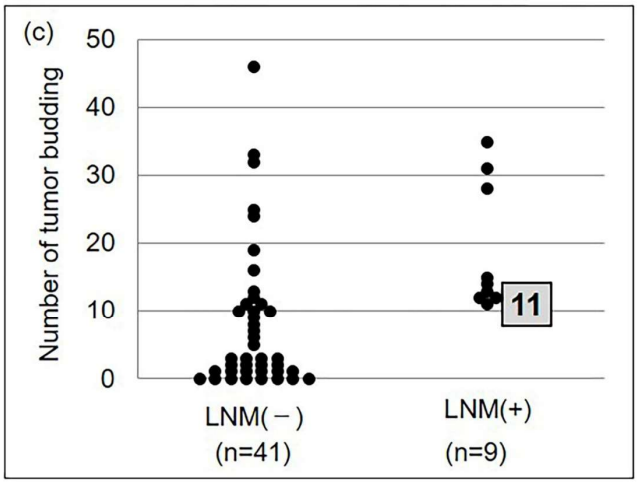
Fig. 2

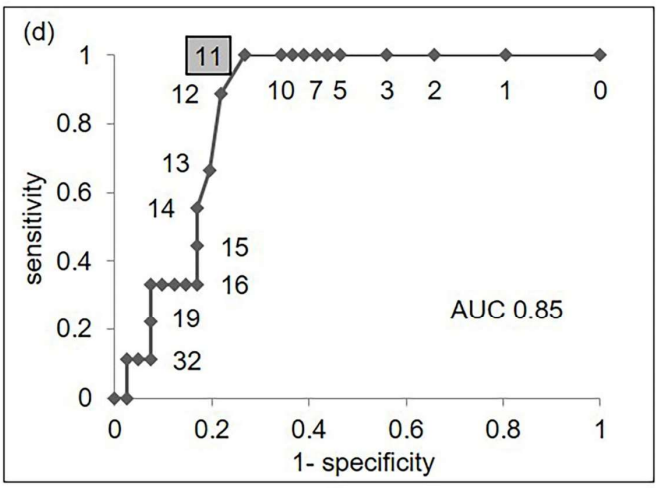
HE staining





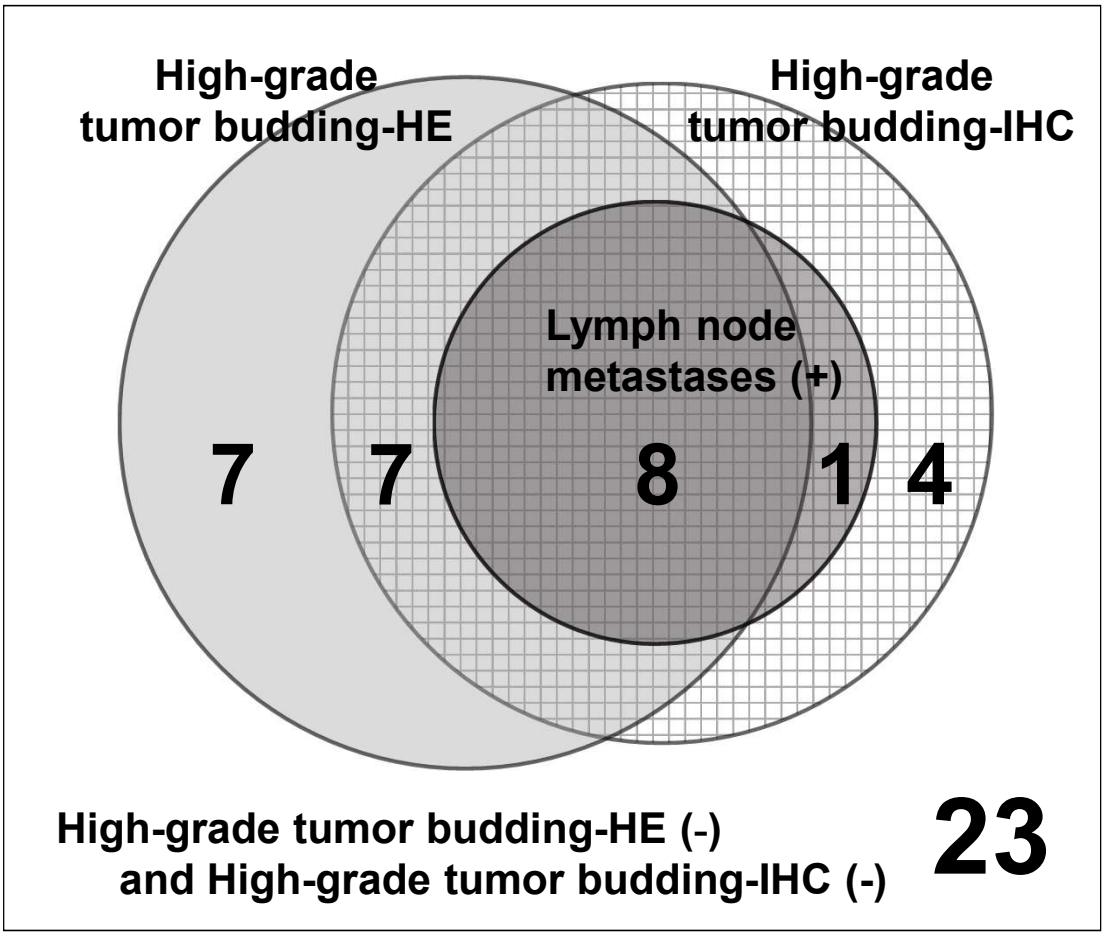
Cytokeratin 5/6 immunohistochemistry





LNM: Lymph node metastasis, AUC: Area under curve

Fig. 3


High-grade
tumor budding-HE
High-grade
tumor budding-IHC
Lymph node
metastases (+)
7
7
8
1
4
High-grade tumor budding-HE (-)
and High-grade tumor budding-IHC (-)
23