Diseases of the Esophagus (2010) **23**, 398–407
DOI: 10.1111/j.1442-2050.2009.01023.x

DISEASES OF THE ESOPHAGUS

Original article

Independent histological risk factors for lymph node metastasis of superficial esophageal squamous cell carcinoma; implication of claudin-5 immunohistochemistry for expanding the indications of endoscopic resection

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SUMMARY. **Endoscopic resection is curative for superficial esophageal squamous cell carcinoma (ESCC) limited to the lamina propria. Endoscopic resection is not recommended for superficial ESCC invading muscularis mucosa or submucosa, however, because of the high frequency of lymph node metastasis (LNM) in such patients. Methods to more accurately predict LNM by analysis of endoscopically resected specimens are needed. Patients with superficial ESCC who underwent surgery without prior chemoradiotherapy ($n = 110$) were retrospectively examined to determine whether LNM correlated with immunohistochemical parameters and conventional histological parameters, including depth of invasion and vascular permeation. Cancer cell expression of claudins-1, 5, and 7, E-cadherin, β-catenin, and matrix metalloproteinase 7 was evaluated. Univariate analysis revealed that LNM correlated with claudin-5 expression, but not any other immunohistochemical parameter examined. Multivariate analysis revealed three independent risk factors for LNM: aberrant claudin-5 expression in cancer cells (odds ratio; OR [95% confidence interval] = 4.61[1.44–14.77]), depth of submucosal invasion greater than 200 μm (3.55 [1.02–13.17]), and positive lymphatic permeation (3.34 [1.22–9.15]). LNM was found in one of 29 (3.4%) patients with none of these three risk factors, and in 32 of 81 (39.5%) patients with one or more of these risk factors. In superficial ESCC, routine analysis of claudin-5 expression in cancer cells together with depth of invasion and lymphatic permeation may be useful for predicting LNM and thereby reducing the number of patients undergoing additional surgery after successful endoscopic resection.**

KEY WORDS: **claudin-5, esophagus, lymph node metastasis, squamous cell carcinoma, submucosal invasion.**

INTRODUCTION

Squamous cell carcinoma accounts for more than 90% of esophageal cancer in Japan.[1,2] When esophageal squamous cell carcinoma (ESCC) is confined to the mucosa or the submucosa, the term superficial ESCC is used irrespective of lymph node metastases (LNM).[3] In superficial ESCC, curative endoscopic resection can be achieved when the tumor is intraepithelial or the tumor invasion is limited to the lamina propria mucosa, because the frequency of LNM is generally low (0 to 5.6%) in patients with such early lesions.[4–6] On the other hand, endoscopic resection is not recommended for superficial ESCC invading the muscularis mucosa or submucosa[2,6] because the frequency of LNM is generally high (19 to 50%) in patients with such invasive lesions.[7,8]

Although surgical resection of superficial ESCC provides excellent results, patients often complain of reflux, dysphagia, and severe chest pain after esophagectomy. In Japan, to avoid unnecessary surgery, endoscopic resection has recently become the first-choice treatment for superficial ESCC. Additional surgery may then be considered based on histological assessment of the specimens for the risk factors of LNM, such as depth of invasion and vascular permeation. To reduce the number of patients who undergo unnecessary surgery and to increase the number of patients who can be followed without

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additional surgery after endoscopic intervention, more accurate methods to predict LNM by analysis of the endoscopically resected specimens are needed.

Depth of invasion and vascular permeation are currently considered the major risk factors for LNM of ESCC.[2,8,9] Several other histological factors, including infiltrative growth pattern[10–12]; downregulation of cell adhesion molecules, such as E-cadherin[12] and β-catenin[13]; downregulation of tight junction-associated proteins, such as claudin-7[14]; and expression of matrix metalloproteinases (MMP) such as MMP-7[15,16], are recently proposed predictive risk factors for LNM of ESCC. The correlations between LNM and these risk factors, except for infiltrative growth pattern[10] and MMP-7,[15] however, have not been evaluated in superficial ESCC.

Claudins-1 and 7 are tight junction proteins of normal esophageal squamous epithelium,[17] whereas claudin-5 is a tight junction protein of normal endothelial cells.[18] Claudin-5, forming the backbone of tight junctions in blood vessels and lymphatic endothelial cells, has an important role in maintaining the homeostasis of the tissue microenvironment.[19–21] Claudin-5 seems to be important for blood brain barrier function because drug transport across the blood brain barrier in claudin-5-deficient mice is increased.[22] On the other hand, several studies about significance of claudin-5 expression in cancer cells were reported.[23,24] However, the relationship between claudin-5 expression in cancer cells of superficial ESCC and LNM has not been studied.

The aim of the present study was to determine independent risk factors for LNM that can be evaluated by histological analysis of endoscopically resected specimens. Previously estimated risk factors, including depth of invasion, vascular permeation, infiltrative growth pattern, cancer cell expression of β-catenin, E-cadherin, claudins-1 and 7, and MMP-7, were evaluated by retrospective analysis of 110 patients with superficial ESCC who underwent surgery. Aberrant expression of claudin-5 in cancer cells was also evaluated as a possible risk factor for LNM of superficial ESCC.

MATERIALS AND METHODS

Patients and lesions

Superficial ESCC was defined as carcinoma limited within the mucosa or submucosa irrespective of LNM. A total of 110 consecutive patients with superficial ESCC undergoing esophagectomy and lymph node dissection without prior chemoradiotherapy at the Tokyo Medical and Dental University Hospital between August 1992 and January 2008 were retrospectively enrolled in the study. All patients underwent esophagectomy for the primary tumor with three exceptional cases. The three patients underwent endoscopic resection of the primary tumor prior to esophagectomy. Clinicopathological profiles of the patients were analyzed according to the classification of esophageal cancer proposed by the Japanese Society of Esophageal Diseases.[4,5]

Hematoxylin–eosin (HE)-stained sections were reviewed and one or two paraffin blocks that included the most invasive area of the primary tumor were selected. Semiserial sections 4 μm thick were cut from the paraffin-embedded lesions and mounted on Superfrost/MAS coated slides (Matsunami Glass Industries, Ltd., Osaka, Japan). One of the sections was used for Elastica van Gieson staining to evaluate venous permeation. Others were used for the immunohistochemistry described below. Histological factors including depth of invasion, tumor differentiation, tumor growth pattern and vascular permeation, and immunohistochemical results were evaluated by two independent observers (T. C. and H. K.) without knowledge of the clinical outcomes. All for which there was disagreement between the two observers were reevaluated and complete agreement was reached after discussion.

Depth of invasion

According to the Japanese classification of esophageal cancer,[4,5] depth of invasion was classified as T1a-EP (in situ), T1a-LPM (invading the lamina propria), T1a-MM (invading the muscularis mucosa), and T1b (submucosal invasion). According to the depth of submucosal invasion, T1b tumors were further classified into two groups; T1b-shallow (submucosal invasion 200 μm or less) and T1b-deep (submucosal invasion greater than 200 μm) (Fig. 1). The cutoff line between the two T1b groups was based on the study by Yoshida *et al.*[2] Depth of submucosal invasion (distance from muscularis mucosa to the submucosal invasive front) was measured using a scale under microscopy. Desmin immunostaining was used to identify the muscularis mucosa.

Tumor differentiation

Tumor differentiation was classified into three groups: well differentiated, moderately differentiated, and poorly differentiated, based on the World Health Organization criteria for histological classification.[3]

Vascular permeation

Vascular (lymphatic and venous) permeation was evaluated as positive or negative using semiserial sections of HE and Elastica van Gieson staining supported by D2-40 immunostaining for lymphatic endothelial cells and CD34 immunostaining for venous endothelial cells.

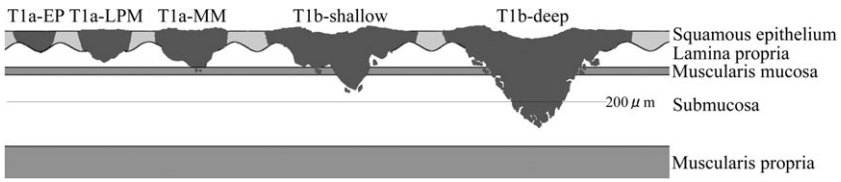

Fig. 1 Classification of superficial esophageal squamous cell carcinoma according to depth of invasion. Cancer lesions were classified as T1a-EP (intra-epithelial cancer), T1a-LPM (invading the lamina proprial mucosa), T1a-MM (invading the muscularis mucosa), and T1b (submucosal invasion). For T1b tumors, depth of submucosal invasion was determined by measuring the vertical distance between the muscularis mucosa and invasive front of the tumor. A T1b-shallow tumor was defined as a lesion with submucosal invasion of 200 μm or less and a T1b-deep tumor as a lesion with submucosal invasion of more than 200 μm.

Tumor growth pattern

Tumor growth pattern at the invasive front was evaluated on HE-stained sections according to the method by Egashira *et al.*[10]; a cancer cell nest 20 μm or less in maximum diameter and free from primary foci connected to intraepithelial cancers on the section was defined as a droplet infiltration. The tumors were considered positive when the lesions contained one or more droplet infiltration.

Immunostaining

Immunostaining was performed according to the protocol described in each reference paper with modifications. The antibodies used in the study are listed in Table 1, including their manufacturers, antigen retrieval method, buffer pH for the retrieval, working dilution of primary antibody, incubation time and temperature, and references.[18,25–31] Endogenous peroxidase activity was blocked by 10 min of incubation with 0.03% hydrogen peroxide solution containing 10 mM sodium azide. All sections were incubated with a primary antibody at its working dilution for 60 min at room temperature or 24 h at 4C, subsequent to antigen retrieval. Sections were stained to detect each antigen using a Vectastain ABC immunoperoxidase kit (Vector Laboratories, Burlington, VT, USA) or an EnVision+ System (Dako A/S, Glostrup, Denmark). The sections were incubated for 10 min in 3,3′-diaminobenzidine, which stained the antigen brown, and counterstained with Mayer's hematoxylin.

Evaluation of immunostaining

Immunostaining was evaluated at the areas of tumor invasion. Based on previously published criteria,[32,33] the cancer cell expression of E-cadherin and β-catenin was compared with that of normal epithelial cells, which constitutively express these molecules, in the same samples. Cancer cells whose staining was stronger than or as strong as that of normal epithelial cells were classified as positive. If the staining was weak or negative, the cells were classified as negative. The expression of E-cadherin and β-catenin in the tumors was graded according to the proportion of positive cells. When 90% or more of the cancer cells were positively stained, the tumors were considered to have preserved expression. When less than 90% of cancer cells were positively stained, the tumors were considered to have reduced expression (Fig. 2).

The immunostaining signals of cytoplasmic β-catenin or MMP-7 were scored as the number of stained cells divided by the total number of cancer cells, and the expression of these cytoplasmic proteins in the tumors was considered positive when more than 10% of the cancer cells were stained (Fig. 2), according to the criteria by Saeki *et al.*[34]

Table 1 Antibodies used in the study

Antibody to	Manufacturers	Working dilution	Antigen retrieval	Buffer pH	Incubation time and temperature	Second antibody	Reference number
Desmin	DakoCytomation, Glostrup, Denmark	1 : 100	MW, 40 min	9.0	1 h, RT	EnVision	[25]
D2-40	Covance, Princeton, NJ, USA	1 : 50	MW, 40 min	9.0	1 h, RT	EnVision	[26]
CD34	Nichirei, Tokyo, Japan	1 : 1	None	–	24 h, 4C	EnVision	
E-cadherin	DakoCytomation, Glostrup, Denmark	1 : 100	AC, 20 min	6.0	24 h, 4C	ABC	[28]
β-catenin	DakoCytomation, Glostrup, Denmark	1 : 100	AC, 20 min	6.0	24 h, 4C	EnVision	[27]
MMP-7	Daiichi Fine Chemical, Toyama, Japan	1 : 2500	MW, 20 min	9.0	24 h, 4C	ABC	[29]
Claudin-1	Zymed, San Francisco, CA, USA	1 : 500	AC, 20 min	9.0	24 h, 4C	ABC	[18]
Claudin-5	Zymed, San Francisco, CA, USA	1 : 80	MW, 40 min	9.0	1 h, RT	ABC	[30]
Claudin-7	Zymed, San Francisco, CA, USA	1 : 100	AC, 20 min	9.0	24 h, 4C	ABC	[31]

ABC, ABC immunoperoxidase kit (Vector Laboratories); AC, autoclave; EnVision, EnVision + System (DAKO A/S); MW, microwave; RT, room temperature.

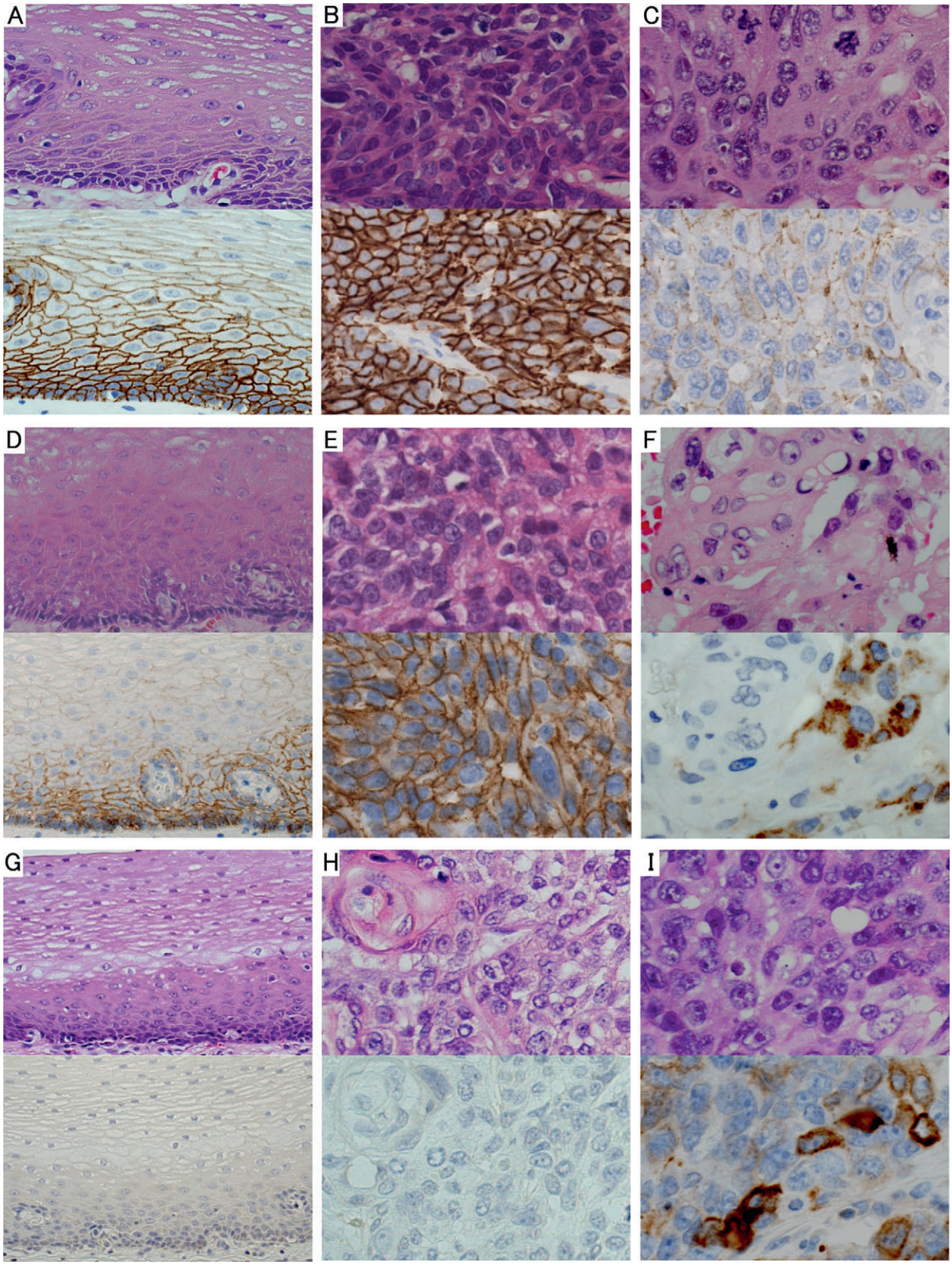

Fig. 2 Cancer cell expression of E-cadherin, β-catenin, and matrix metalloproteinases (MMP)-7. Immunostaining patterns of E-cadherin (A, B, and C), β-catenin (D, E, and F), and MMP-7 (G, H, and I) are shown together with hematoxylin–eosin staining of each semiserial section (upper half). Membrane staining of E-cadherin and β-catenin was observed in the parabasal cells of the normal esophageal epithelial layer (A and D). Such staining patterns were preserved in some cancer lesions (B and E) and reduced in the other cancer lesions (C). Cytoplasmic expression of β-catenin was observed in some lesions (F). MMP-7 was not expressed in normal esophageal epithelium (G). Cancer cell expression of MMP-7 was observed in some lesions (I), whereas MMP-7 was totally negative in the other lesions (H).

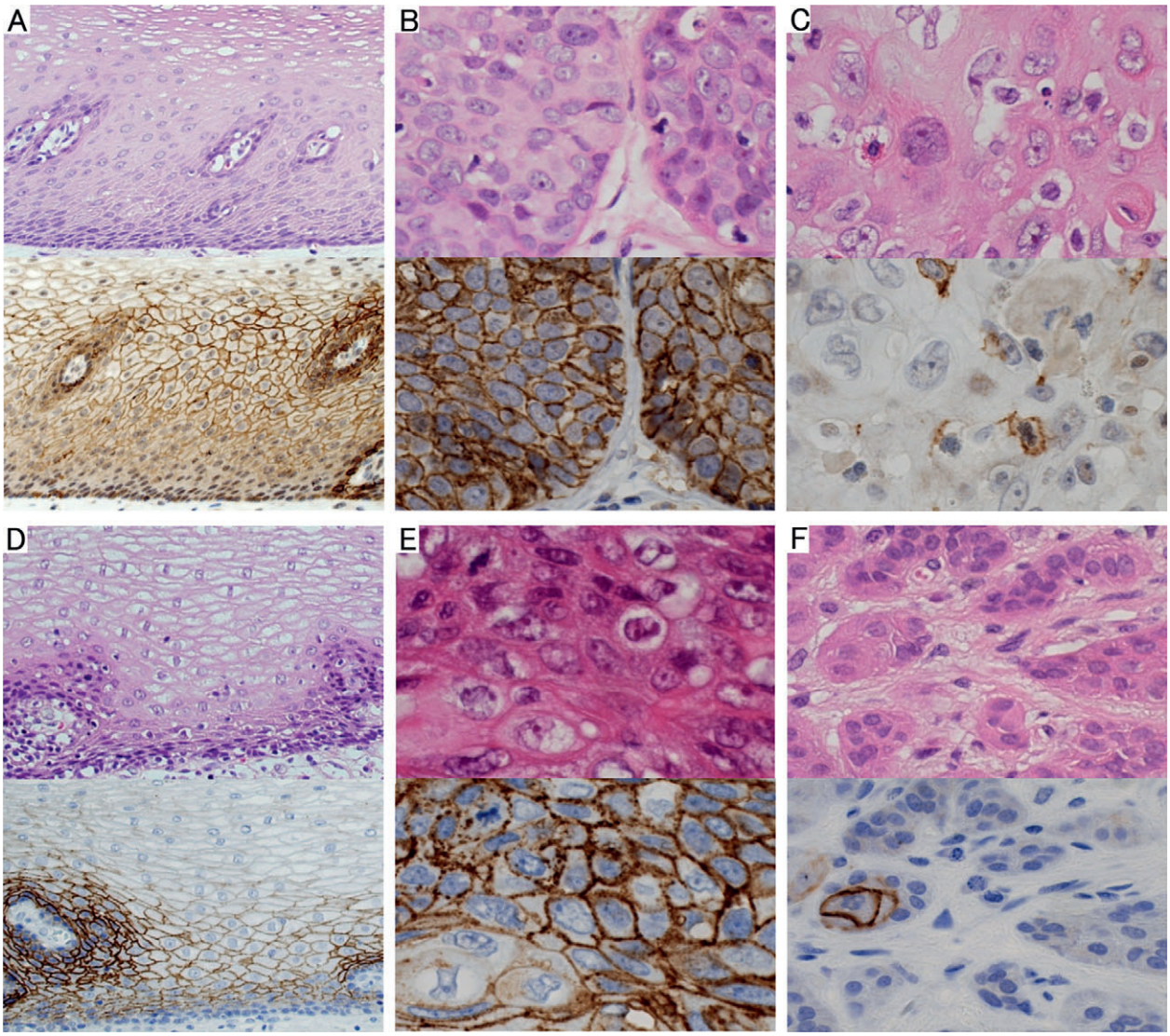

Fig. 3 Reduced or preserved cancer cell expression of claudins-1 and 7. Immunostaining patterns of claudin-1 (A, B, and C) and claudin-7 (D, E, and F) are shown together with hematoxylin–eosin staining of each semiserial section (upper half). Membrane staining of claudins-1 and 7 was observed in the prickle cells of the normal esophageal epithelial layer (A and D). Such staining patterns were preserved in some cancer lesions (B and E) and reduced in other cancer lesions (C and F).

In the study, reduced expression of membranous β-catenin and abnormal accumulation of cytoplasmic β-catenin in cancer cells were evaluated independently because each event is considered to be important in terms of alteration in Wnt signaling pathway.[13]

Membrane staining of claudins-1 and 7 was classified as positive when the intensity of the cancer cells was equivalent to that in normal squamous cells and scored as the number of positive cells divided by the total number of cancer cells. The expression of claudins-1 and 7 in the tumors was considered positive when more than 25% of the cancer cells were stained (Fig. 3), according to the criteria by Takala *et al.*[23] Because claudin-5 was not expressed at all in normal squamous epithelium, cancer cell expression of claudin-5 was considered positive when any of the cancer cells in the tumors were stained (Fig. 4).

Statistical analysis

For univariate and multivariate analysis of LNM, a logistic regression model was used. To fit the multivariate models, a forward conditional method was used to introduce variables stepwise into the model. The chi-square test was used to evaluate the correlation between two different groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Univariate analysis for LNM

In the study, LNM was detected in 33 (30.0%) of the 110 patients. The correlation between LNM and clinicopathological and immunohistochemical

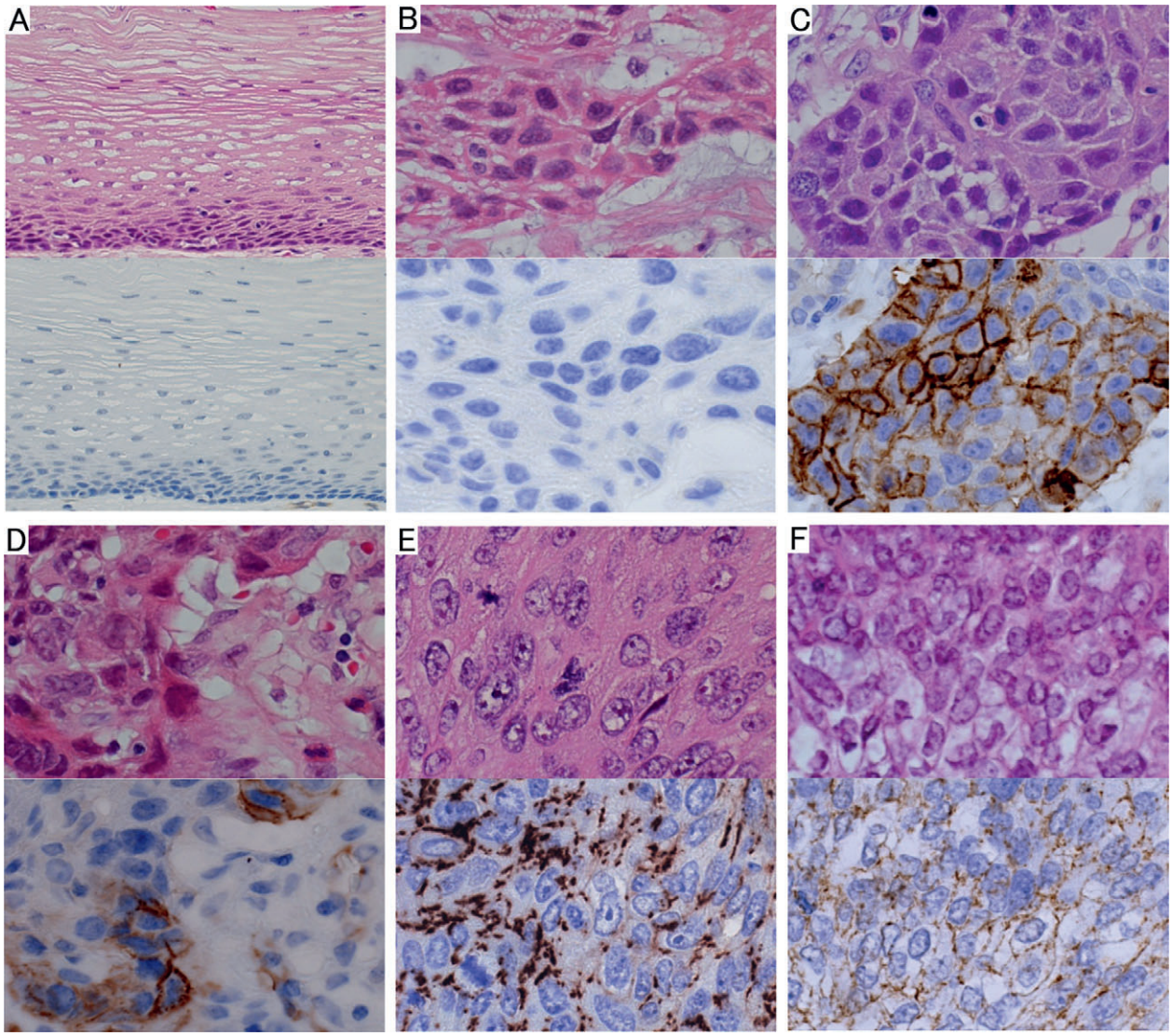

Fig. 4 Aberrant cancer cell expression of claudin-5. Representative immunostaining patterns of claudin-5 in cancer cells are shown together with hematoxylin–eosin staining of each semiserial section (upper half). Claudin-5 was not expressed in normal esophageal epithelium (A). Claudin-5 was totally negative in tumors of many patients (B). In tumors with aberrant claudin-5 expression (C, D, E, and F), the intensity and distribution of claudin-5-positive cancer cells were variable. The membrane staining pattern of claudin-5 was either diffuse (C) or focal (D). Discontinuous pericellular staining was prominent in some samples (E) and scattered in other samples (F).

parameters was examined by univariate analysis. LNM correlated with clinicopathological parameters such as female ($P = 0.007$), poorly differentiated type ($P = 0.047$), depth of submucosal invasion greater than 200 μm ($P < 0.001$), positive lymphatic permeation ($P < 0.001$), and positive venous permeation ($P < 0.001$; Table 2). LNM was also correlated with immunohistochemical parameter, such as aberrant claudin-5 expression in cancer cells ($P = 0.0074$), but not with any other immunohistochemical parameters examined (Table 3).

Multivariate analysis for LNM

The five histological parameters that were significantly correlated with LNM in univariate analysis were used for the logistic regression model (Table 4). LNM independently correlated with aberrant claudin-5 expression in cancer cells, depth of submucosal invasion greater than 200 μm, and positive lymphatic permeation.

Frequency of LNM according to the number of independent risk factors

Frequency of LNM was evaluated according to the number of independent risk factors revealed by multivariate analysis. The evaluation was performed with the two risk factors already used in conventional routine examination (depth of submucosal invasion greater than 200 μm and positive lymphatic permeation) (Table 5) and with all three independent risk factors including aberrant claudin-5 expression in cancer cells (Table 6). The frequency of LNM

Table 2 Univariate analysis of clinicopathological parameters for lymph node metastasis

Clinicopathological parameters	Number of patients (n = 110)	Lymph node metastasis (%)	P-value
Sex			
Male	98	25 (25.5)	0.0070
Female	12	8 (66.7)	
Age			
Less than 60	37	10 (27.0)	0.63
60 or more	73	23 (31.5)	
Treatment			
Esophagectomy (only)	107	31 (29.0)	0.16
EMR or ESD + esophagectomy	3	2 (66.7)	
Differentiation			
Well + moderately differentiated	72	17 (23.6)	0.047
Poorly differentiated	38	16 (42.1)	
Depth of invasion			
T1a	33	3 (9.1)	
T1a-EP	0	–	
T1a-LPM	2	0 (0.0)	
T1a-MM	31	3 (9.7)	
T1b	77	30 (39.0)	
T1b-shallow	13	2 (15.4)	
T1b-deep	64	28 (43.8)	<0.001*
T1a +T1b-shallow	46	5 (10.9)	
Droplet infiltration			
Positive	77	25 (32.5)	0.39
Negative	33	8 (24.2)	
Lymphatic permeation			
Positive	46	22 (47.8)	<0.001
Negative	64	11 (17.2)	
Venous permeation			
Positive	40	20 (50.0)	<0.001
Negative	70	13 (18.6)	

*Univariate analysis was performed for T1b-deep vs. T1a + T1b-shallow. EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; T1a, intramucosal carcinoma; T1a-EP, carcinoma in situ; T1a-LPM, carcinoma invading the lamina propria; T1a-MM, carcinoma invading the muscularis mucosa; T1b, carcinoma with submucosal invasion; T1b-shallow, carcinoma with submucosal invasion 200 μm or less; T1b-deep, carcinoma with submucosal invasion greater than 200 μm.

Table 3 Univariate analysis of immunohistochemical parameters for lymph node metastasis

Immunohistochemical parameters	Number of patients (n = 110)	Lymph node metastasis (%)	P-value
E-cadherin			
Reduced	59	19 (32.2)	0.59
Preserved	51	14 (27.4)	
β-catenin (membranous)			
Reduced	63	20 (31.4)	0.65
Preserved	47	13 (27.7)	
β-catenin (cytoplasmic)			
Positive	41	12 (29.3)	0.90
Negative	69	21 (30.4)	
Matrix metalloproteinase 7			
Positive	19	7 (36.8)	0.48
Negative	91	26 (28.6)	
Claudin-1			
Negative	10	1 (10.0)	0.15
Positive	100	32 (32.0)	
Claudin-5			
Positive	17	10 (58.8)	0.0074
Negative	93	23 (24.3)	
Claudin-7			
Negative	19	8 (42.1)	0.21
Positive	91	25 (27.5)	

Table 4 Multivariate analysis of the five histological parameters for lymph node metastasis

Parameters	OR	(95% CI)	*P*-value
Aberrant expression of claudin-5 in cancer cells	4.61	(1.44–14.77)	0.010
Depth of submucosal invasion greater than 200 μm	3.55	(1.02–13.17)	0.048
Positive lymphatic permeation	3.34	(1.22–9.15)	0.019
Poorly differentiated cancer	2.36	(0.87–6.39)	0.081
Positive venous permeation	1.62	(0.52–5.04)	0.40

OR, odds ratio; 95% CI, 95% confidence interval.

increased according to the number of risk factors in both types of combination. By adding aberrant claudin-5 expression as a new risk factor, the frequency of LNM increased in patients with all risk factors (60.0% vs. 87.5%) and decreased in patients with no risk factor (9.1% vs. 3.4%).

DISCUSSION

In the present study, claudin-5 expression was detected in the cancer cells in some patients. Multivariate analysis revealed three independent histological risk factors for LNM: aberrant claudin-5 expression in cancer cells, depth of submucosal invasion greater than 200 μm, and positive lymphatic permeation. Therefore, histological evaluation of claudin-5 expression in cancer cells, depth of invasion, and lymphatic permeation during examination of specimens obtained by successful endoscopic resection of superficial ESCC may be useful for predicting LNM and may help to reduce the number of patients who undergo additional surgery after successful endoscopic resection.

Table 5 Frequency of lymph node metastasis according to the number of the two risk factors already used in routine examination

Number of the risk factors†	Number of patients with the risk factors	Number (%) of patients with nodal metastasis
One or more	77	30 (39.0)*
2	35	21 (60.0)
1	42	9 (21.4)
None	33	3 (9.1)*

*$P = 0.0011$. †Risk factors include submucosal invasion greater than 200 μm and positive lymphatic permeation.

Table 6 Frequency of lymph node metastasis according to the number of the three independent risk factors

Number of the risk factors†	Number of patients with the risk factors	Number (%) of patients with nodal metastasis
One or more	81	32 (39.5)*
3	8	7 (87.5)
2	30	15 (50.0)
1	43	10 (23.3)
None	29	1 (3.4)*

*$P < 0.001$. †Risk factors include aberrant expression of claudin-5 in cancer cells, submucosal invasion greater than 200 μm, and positive lymphatic permeation.

LNM was independently correlated with claudin-5 expression in cancer cells. Dendritic cells are equipped with tight junction proteins that enable them to migrate into the luminal space by passing through the mucosal layer composed of epithelial cells expressing identical tight junction proteins.[35,36] Cancer cells with aberrant claudin-5 expression may tend to colonize in the lymphatic sinus because claudin-5-positive cancer cells have an affinity for the sinus-lining endothelial cells of lymph nodes that express the specific molecular architecture of cellular junctions, including claudin-5.[37] Alternatively, aberrant expression of claudin-5 may reflect the malignant potential of cancer cells because claudin-5 regulates cell proliferation in ESCC[23] and gastric cancer.[24]

Takala *et al.* reported that LNM did not correlate with cancer cell expression of claudin-5 in esophageal cancer.[23] The discrepancy between their study and ours may be due to differences in the cases examined. We examined only cases of superficial ESCC, whereas they examined 54 cases of ESCC and 20 cases of esophageal adenocarcinoma. Claudin-5 expression was more frequent in esophageal adenocarcinoma (53%) than ESCC (25%). Furthermore, their study included many cases of advanced ESCC in which LNM seems to be more frequent than in superficial ESCC.

Because claudin-5 is not expressed in normal squamous epithelium of the esophagus, aberrant expression of this protein can be easily and subjectively evaluated. Any membrane immunostaining of cancer cells in the tumors was considered aberrant expression, regardless of the intensity and distribution of positive signals in the tumor. This is why we propose that this immunohistochemical parameter can be used together with depth of invasion and lymphatic permeation in routine examination to estimate the risk for LNM. LNM was found in one (3.4%) of the 29 patients with none of these three risk factors, whereas LNM was found in 32 (39.5%) of the 81 patients with one or more of these risk factors.

In the present study of superficial ESCC, LNM was not correlated with parameters associated with cancer cell-to-cell interactions, including reduced expression of adhesion molecules such as E-cadherin and β-catenin and tight junction molecules such as claudin-1 and claudin-7. In some reports, reduced cancer cell expression of E-cadherin correlated with LNM in ESCC.[12,13] In ESCC, LNM correlated with the reduced expression of membranous β-catenin[38] and the aberrant expression of cytoplasmic β-catenin.[12] Aberrant cancer cell expression of

MMP-7 also correlated with LNM in ESCC.[16] Usami *et al.* reported that reduced cancer cell expression of claudin-7, but not claudin-1, correlated with LNM in ESCC.[14] In the present study, the cutoff points between normal and abnormal expressions for these parameters were decided by using definitions of the previous studies.[23,32–34] Even when LNM was evaluated by using other cutoff points with the help of a receiver operating characteristic (ROC) curve, statistical significance could not be found between LNM and abnormal expressions (data not shown). All of the previous studies which reported significant correlations between LNM and abnormal expressions included many cases with invasion of the proprial muscle layers or even deeper. In contrast to these studies, the present study included only cases of superficial ESCC to clarify the risk factors for LNM in early lesions that can be treated by endoscopic resection. Therefore, direct comparison of the statistical findings between these previous reports and our results is not relevant for these immunohistochemical parameters.

Lymphatic permeation was an independent risk factor, but the odds ratio was not higher than the other two independent risk factors revealed in the study. Although vascular permeation, especially lymphatic permeation, is believed to be useful for predicting LNM, LNM occurs in many cases without lymphatic permeation. Eguchi *et al.* reported that LNM occurs in 10% of T1a-MM tumors without lymphatic permeation.[9] In the present study, LNM was found in 9% of T1a-MM (17% of T1b-shallow and 20% of T1b-deep) tumors without lymphatic permeation. Thus, this parameter is not a reliable predictive factor unless the other risk factors are considered at the same time.

Much interest has been paid to the infiltration pattern of cancer cells at the invasive front of gastrointestinal cancer. In colorectal cancer, small cancer cell nests, called 'tumor budding' or 'sprouting,' are a major predictive risk factor for LNM in early invasive cancer[39] and for a poor survival rate in advanced cancer.[40] In superficial ESCC, such an infiltrative growth pattern of cancer cells has been named droplet infiltration, emphasizing its significant correlation with LNM in T1a-MM tumors.[10] In the present study, however, droplet infiltration did not correlate with LNM, even when limited to T1a-MM tumors. In fact, some of the lesions without droplet infiltration directly invaded the lymphatic vessels, forming relatively larger cell nests. Further studies are needed to conclude whether droplet infiltration is predictive of LNM.

It is widely accepted that endoscopic resection of superficial ESCC lesions with T1a-EP and T1a-LPM can be curative because these lesions lack LNM,[2,6] but the present study did not provide any information about this point because there were no cases of T1a-EP and only two cases of T1a-LPM. LNM was found in 10% of esophageal T1a-MM cancer in the study. On the other hand, LNM is extremely rare in intramucosal gastric cancer,[41,42] and colorectal cancer.[39,43] The frequency of LNM is reported to be 20 or 18% in early invasive gastric cancer[41,42] and 11 or 10% in early invasive colorectal cancer,[39,43] whereas the frequency is reported to be 30 or 57% in superficial ESCC with submucosal invasion (T1b).[7,8] In the present study, LNM frequency was 39% in all T1b (15% in T1b-shallow and 44% in T1b-deep), which is consistent with the previous reports. Especially after successful endoscopic resection of superficial ESCC of T1a-MM and T1b, the risk factors for LNM need to be evaluated in the specimens to determine whether additional surgery should be recommended to remove lymph nodes with possible metastasis.

In the present study, we did a retrospective analysis of patients with superficial ESCC who underwent esophagectomy and lymph node dissection. The results obtained here indicate that it may be possible to evaluate the risk for LNM in patients who are first treated by endoscopic resection for superficial ESCC. In the absence of such evaluation, even if the patients examined here had been first treated by endoscopic resection before surgery, additional surgery may have been performed in almost all patients, except for the two patients with a T1a-LPM type tumor, and metastasis would not have been detected in 69% (75/108) of the patients undergoing additional surgery. Using the histological evaluation proposed here, the number of such patients who underwent unnecessary surgery may have been decreased and, at the same time, the number of patients who could be followed without additional surgery may have been increased.

There are a few studies[7–10,44,45] in which the incidence of LNM has been reported for superficial ESCC. More data on the risk factors for LNM of superficial ESCC are needed to determine the best therapeutic strategy of superficial ESCC. The indication for endoscopic mucosal resection and endoscopic submucosal dissection may be expanded by immunohistochemical evaluation of claudin-5 expression in cancer cells, together with conventional histological evaluation for depth of invasion and lymphatic permeation.

References

1 Japan Esophageal Society. Comprehensive registry of esophageal cancer in Japan. 3rd edition. 2002 [Cited May 1, 2009.] Available from URL: http://esophagus.jp
2 Takubo K, Aida J, Sawabe M *et al.* Early squamous cell carcinoma of the esophagus: the Japanese viewpoint. Histopathology 2007; 51: 733–42.
3 Hamilton S, Aaltonen L, (eds). World Health Organization Classification of Tumors. Pathology and Genetics of Tumors of the Digestive System. Lyon: IARC Press, 2000.

4 Japanese Society for Esophageal Diseases. Guidelines for clinical and pathologic studies on carcinoma of the esophagus, ninth edition: preface, general principles, part I. Esophagus 2004; 1: 61–88.
5 Japanese Society for Esophageal Diseases. Guidelines for clinical and pathologic studies on carcinoma of the esophagus, ninth edition: part II. Esophagus 2004; 1: 107–26.
6 Endo M, Yoshino K, Kawano T, Nagai K, Inoue H. Clinicopathologic analysis of lymph node metastasis in surgically resected superficial cancer of the thoracic esophagus. Dis Esophagus 2000; 13: 125–9.
7 Araki K, Ohno S, Egashira A, Saeki H, Kawaguchi H, Sugimachi K. Pathologic features of superficial esophageal squamous cell carcinoma with lymph node and distal metastasis. Cancer 2002; 94: 570–5.
8 Tomita N, Matsumoto T, Hayashi T *et al.* Lymphatic invasion according to D2-40 immunostaining is a strong predictor of nodal metastasis in superficial squamous cell carcinoma of the esophagus: algorithm for risk of nodal metastasis based on lymphatic invasion. Pathol Int 2008; 58: 282–7.
9 Eguchi T, Nakanishi Y, Shimoda T *et al.* Histopathological criteria for additional treatment after endoscopic mucosal resection for esophageal cancer: analysis of 464 surgically resected cases. Mod Pathol 2006; 19: 475–80.
10 Egashira H, Yanagisawa A, Kato Y. Predictive factor for lymph node metastasis in esophageal squamous cell carcinomas contacting or penetrating the muscularis mucosae: the utility of droplet infiltration. Esophagus 2006; 3: 47–52.
11 Chibana Y, Fujii S, Ichikawa K *et al.* Tumor cell dissociation score highly correlates with lymph node metastasis in superficial esophageal carcinoma. J Gastroenterol Hepatol 2005; 20: 1371–8.
12 Natsugoe S, Uchikado Y, Okumura H *et al.* Snail plays a key role in E-cadherin-preserved esophageal squamous cell carcinoma. Oncol Rep 2007; 17: 517–23.
13 Zhang G, Zhou X, Xue L *et al.* Accumulation of cytoplasmic beta-catenin correlates with reduced expression of E-cadherin, but not with phosphorylated Akt in esophageal squamous cell carcinoma: immunohistochemical study. Pathol Int 2005; 55: 310–7.
14 Usami Y, Chiba H, Nakayama F *et al.* Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. Hum Pathol 2006; 37: 569–77.
15 Tanioka Y, Yoshida T, Yagawa T *et al.* Matrix metalloproteinase-7 and matrix metalloproteinase-9 are associated with unfavourable prognosis in superficial oesophageal cancer. Br J Cancer 2003; 89: 2116–21.
16 Yamashita K, Mori M, Shiraishi T, Shibuta K, Sugimachi K. Clinical significance of matrix metalloproteinase-7 expression in esophageal carcinoma. Clin Cancer Res 2000; 6: 1169–74.
17 Lioni M, Brafford P, Andl C, *et al.* Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. Am J Pathol 2007; 170: 709–21.
18 Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci U S A 1999; 96: 511–6.
19 Matter K, Balda M S. Holey barrier: claudins and the regulation of brain endothelial permeability. J Cell Biol 2003; 161: 459–60.
20 Neuhaus W, Wirth M, Plattner V E, Gabor F, Noe C R. Expression of Claudin-1, Claudin-3 and Claudin-5 in human blood-brain barrier mimicking cell line ECV304 is inducible by glioma-conditioned media. Neurosci Lett 2008; 446: 59–64.
21 Mays T A, Binkley P F, Lesinski A *et al.* Claudin-5 levels are reduced in human end-stage cardiomyopathy. J Mol Cell Cardiol 2008; 45: 81–7.
22 Nitta T, Hata M, Gotoh S *et al.* Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003; 161: 653–60.
23 Takala H, Saarnio J, Wiik H, Soini Y. Claudins 1, 3, 4, 5 and 7 in esophageal cancer: loss of claudin 3 and 4 expression is associated with metastatic behavior. APMIS 2007; 115: 838–47.
24 Soini Y, Tommola S, Helin H, Martikainen P. Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. Virchows Arch 2006; 448: 52–8.
25 Miettinen M, Sarlomo-Rikala M, Sobin L H, Lasota J. Esophageal stromal tumors: a clinicopathologic, immunohistochemical, and molecular genetic study of 17 cases and comparison with esophageal leiomyomas and leiomyosarcomas. Am J Surg Pathol 2000; 24: 211–22.
26 Franchi A, Gallo O, Massi D, Baroni G, Santucci M. Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. Cancer 2004; 101: 973–8.
27 Bukholm I K, Nesland J M, Borresen-Dale A L. Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [see comments]. J Pathol 2000; 190: 15–9.
28 Shiozaki H, Tahara H, Oka H *et al.* Expression of immunoreactive E-cadherin adhesion molecules in human cancers. Am J Pathol 1991; 139: 17–23.
29 Ueno H, Yamashita K, Azumano I, Inoue M, Okada Y. Enhanced production and activation of matrix metalloproteinase-7 (matrilysin) in human endometrial carcinomas. Int J Cancer 1999; 84: 470–7.
30 Morita K, Sasaki H, Furuse M, Tsukita S. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 1999; 147: 185–94.
31 Reyes J L, Lamas M, Martin D *et al.* The renal segmental distribution of claudins changes with development. Kidney Int 2002; 62: 476–87.
32 Xiangming C, Hokita S, Nuruki K *et al.* The expression of cadherin-catenin complex in association with the clinicopathologic features of early gastric cancer. Surg Today 1998; 28: 587–94.
33 Xiangming C, Hokita S, Natsugoe S *et al.* Cooccurrence of reduced expression of alpha-catenin and overexpression of p53 is a predictor of lymph node metastasis in early gastric cancer. Oncology 1999; 57: 131–7.
34 Saeki H, Tanaka S, Sugimachi K *et al.* Interrelation between expression of matrix metalloproteinase 7 and beta-catenin in esophageal cancer. Dig Dis Sci 2002; 47: 2738–42.
35 Sung S S, Fu S M, Rose C E Jr, Gaskin F, Ju S T, Beaty S R. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol 2006; 176: 2161–72.
36 Rescigno M, Rotta G, Valzasina B, Ricciardi-Castagnoli P. Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology 2001; 204: 572–81.
37 Pfeiffer F, Kumar V, Butz S *et al.* Distinct molecular composition of blood and lymphatic vascular endothelial cell junctions establishes specific functional barriers within the peripheral lymph node. Eur J Immunol 2008; 38: 2142–55.
38 Zhao X J, Li H, Chen H *et al.* Expression of e-cadherin and beta-catenin in human esophageal squamous cell carcinoma: relationships with prognosis. World J Gastroenterol 2003; 9: 225–32.
39 Ueno H, Mochizuki H, Hashiguchi Y *et al.* Risk factors for an adverse outcome in early invasive colorectal carcinoma. Gastroenterology 2004; 127: 385–94.
40 Ueno H, Price A B, Wilkinson K H, Jass J R, Mochizuki H, Talbot I C. A new prognostic staging system for rectal cancer. Ann Surg 2004; 240: 832–9.
41 Gotoda T, Sasako M, Ono H, Katai H, Sano T, Shimoda T. Evaluation of the necessity for gastrectomy with lymph node dissection for patients with submucosal invasive gastric cancer. Br J Surg 2001; 88: 444–9.
42 Haruta H, Hosoya Y, Sakuma K *et al.* Clinicopathological study of lymph-node metastasis in 1,389 patients with early gastric cancer: assessment of indications for endoscopic resection. J Dig Dis 2008; 9: 213–8.
43 Kitajima K, Fujimori T, Fujii S *et al.* Correlations between lymph node metastasis and depth of submucosal invasion in submucosal invasive colorectal carcinoma: a Japanese collaborative study. J Gastroenterol 2004; 39: 534–43.
44 Kim D U, Lee J H, Min B H *et al.* Risk factors of lymph node metastasis in T1 esophageal squamous cell carcinoma. J Gastroenterol Hepatol 2008; 23: 619–25.
45 Tajima Y, Nakanishi Y, Ochiai A *et al.* Histopathologic findings predicting lymph node metastasis and prognosis of patients with superficial esophageal carcinoma: analysis of 240 surgically resected tumors. Cancer 2000; 88: 1285–93.