J Med Dent Sci 2012; 59: 17–28

Original Article

Reduced expression of cytokeratin 4 and 13 is a valuable marker for histologic grading of esophageal squamous intraepithelial neoplasia

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Histologic evaluation of low-grade or high-grade intraepithelial neoplasia (LG-IN or HG-IN) of the esophagus is important for estimating the risk of progression to invasive carcinoma. Discrimination between LG-IN and HG-IN, or neoplasia and non-neoplastic lesion (NNL), however, is occasionally difficult. This study was designed to evaluate whether cytokeratin expression can be used for discrimination of these lesions. Esophageal Iodine-unstained lesions (n=154), less than 10 mm, were classified into HG-IN, LG-IN, and NNL. These lesions together with 154 foci of normal esophageal epithelium (NEE) were examined by immunohistochemistry for cytokeratins (CK4 and CK13), p53 overexpression, and the MIB-1 labeling index. The ratios of CK4- and CK13-positive staining were scored from 1 to 3. The CK4- and CK13-positive staining ratios were decreased in NNL (73% and 78%), LG-IN (55% and 69%), and HG-IN (33% and 48%), compared to NEE (91% and 95%). The differences between LG-IN and HG-IN, neoplasia and NNL, and among these three lesions and NEE were statistically significant ($p < 0.005$). The cytokeratin scores correlated with the MIB-1 labeling index (both: $p < 0.0001$), but not with p53 overexpression. CK4 and CK13 immunohistochemistry could be an objective method for evaluating the risk for progression to invasive carcinoma.

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Received September 22 ; Accepted November 11, 2011

Key words : esophagus, squamous cell carcinoma, intraepithelial neoplasia, cytokeratin 4, cytokeratin 13.

Introduction

Recent progress in endoscopic diagnosis allows for the detection of several minute neoplastic lesions in the esophageal squamous epithelium. Such minute lesions were previously diagnosed based on their reduced or negative reactivity with iodine used for chromoendoscopy [1, 2], and examined by biopsy for histopathologic diagnosis.

In general, endoscopic resection is performed when the biopsy specimen is histopathologically diagnosed as carcinoma *in situ* or high-grade intraepithelial neoplasia (HG-IN) because of the high possibility of progression to invasive carcinoma. When the biopsy specimen is diagnosed as low-grade intraepithelial neoplasia (LG-IN), however, the risk of progression to invasive carcinoma is lower and patients are followed up by endoscopy [2]. Further, when lesions are diagnosed as non-neoplastic lesions (NNLs), such as inflammatory changes, patients do not need endoscopic treatment and can be followed up conservatively. Therefore, histopathologic diagnosis of biopsy specimens is particularly important for determining appropriate and adequate therapy.

Because histopathologic diagnostic criteria for intraepithelial neoplasia are subjective and obscure, even in the World Health Organization (WHO) classification [2], more objective diagnostic markers are needed for appropriate diagnosis and treatment. Immunohistochemistry (IHC) for p53 overexpression is an objective marker for p53 gene alterations [3-7]. In esophageal cancer, however, p53 gene alteration

occurs in early carcinogenesis [8-10] and does not reflect the risk for progression to invasive cancer. Therefore, we need other objective markers to indicate the risk for progression to invasive cancer.

Abnormal expression of cytokeratin (CK) proteins and its implication in the pathologic diagnosis of squamous neoplastic lesions in the head and neck region have been reported [11-14]. Sakamoto et al. reported that reduced expression of CK4 and CK13 are frequently observed in precancerous dysplastic lesions as well as in early carcinoma of the oral mucosa, and emphasized the implication of such cytokeratins for a precise diagnosis [13]. Reduced expression of these cytokeratins has also been reported in a few studies of invasive esophageal cancers [15-17]. The present study was designed to evaluate the implication of CK4 and CK13 expression levels in the squamous intraepithelial lesions of the esophagus, using specimens with iodine-unstained lesions (IULs) obtained by endoscopic or surgical procedures.

Materials and Methods

Patients and lesions

Specimens with IULs were obtained from 44 patients that underwent endoscopic resection and 9 patients that underwent surgery for esophageal cancer at the Tokyo Medical and Dental University Hospital (Tokyo, Japan). The sufficient explanation about this study protocols were given to all patients and informed consent was obtained prior to this study. This study was performed in accordance with the World Medical Association, the Declaration of Helsinki, and the ethics committee of Tokyo Medical and Dental University approved the protocols.

After endoscopic or surgical resection, all specimens were stained with iodine before and after formalin fixation. All clearly demarcated IULs with a diameter of less than 10 mm were identified by macroscopic examination. After histologic examination, IULs due to invasive carcinoma, erosive change, and ectopic gastric mucosa were excluded from the study. Finally, 154 IULs ranging in size from 2.1 to 9.8 mm in greatest diameter were obtained. In patients of esophageal squamous cell carcinoma, high prevalence of multiple primary carcinomas or multiple IULs has been reported [18-20]. Therefore, the dataset in this study may reflect practical condition in treatment of esophageal cancer patients, although multiple IULs from each patient were included in this study. Further, for comparison, 154 areas of normal esophageal mucosa were selected from 53 specimens with IULs and used as normal esophageal epithelium (NEE) for the study.

Semiserial histologic sections (3 μm - thick) were cut from formalin-fixed and paraffin-embedded tissue samples and mounted on silane-coated slides (Muto Pure Chemicals, Co. Ltd., Tokyo, Japan). The first two sections were stained with hematoxylin–eosin and periodic acid-Schiff for evaluating histologic grade, and other sections were used for IHC. Two independent observers including the first author (M.T.) and experienced pathologist (H.K.) evaluated the histologic grade and IHC results. In case of disagreement between the two observers, the observers re-evaluated the specimens and reached a consensus after discussion.

Histologic evaluation and grading

All IULs were classified into the following three categories: HG-IN, LG-IN, and NNL, according to the criteria for histologic classification of the WHO [2]. Morphologic features of intraepithelial neoplasia include both architectural and cytologic abnormalities. Architectural abnormalities are characterized by disorganization of the epithelium and loss of normal cell polarity. Cytologically, the neoplastic cells exhibit irregular and hyperchromatic nuclei, an increase in the nuclear/cytoplasmic ratio, and increased mitotic activity. Furthermore, intraepithelial neoplasias are classified as LG-IN or HG-IN. In LG-IN, abnormalities are often confined to the lower half of the epithelium, whereas in HG-IN, the abnormal cells also occur in the upper half and exhibit a greater degree of atypia. In the present study, carcinoma *in situ*, according to the Japanese criteria [21-23], was included in the category of HG-IN according to the WHO's two-tier system [2]. IULs with no neoplastic lesions according to the criteria described above were classified as NNL.

IHC

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 the primary antibody, incubation time, and temperature. Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogen peroxide solution containing 10 mM sodium azide for 10 minutes. All sections were incubated with a primary antibody at its working dilution for 60 minutes at room temperature or 24 hours at 4°C, subsequent to antigen retrieval. Sections were stained to detect each antigen using a Vectastain ABC Immunoperoxidase kit (Vector Laboratories, Burlington, CA) or an EnVision+

Table 1. Antibodies used in this study

Antibody to;	Clone	Manufacturers	Working Dilution	Antigen Retrieval	Buffer pH	Incubation Time and Temperature	Second Antibody
CK4	EP1599Y	Epitomics, Burlingame, California, USA	1:200	MW, 97°C, 40min	9.0	1hr, RT	EnVision
CK13	KS-1A3	Novocastra, Newcastle upon Tyne, UK	1:200	MW, 97°C, 40min	9.0	1hr, RT	EnVision
p53	DO-7	Novocastra, Newcastle upon Tyne, UK	1:1000	AC, 121°C, 20min	6.0	24hrs, 4ºC	ABC
Ki-67	MIB1	DakoCytomation, Glostrup, Denmark	1:800	AC, 121°C, 20min	6.0	24hrs, 4ºC	ABC

MW, microwave; AC, autoclave; RT, room temperature; EnVision, EnVision+ System (Dako Cytomation); ABC, ABC immunoperoxidase kit (Vector Laboratories).

System (Dako A/S, Glostrup, Denmark). The sections were incubated for in 3,3'-diaminobenzidine for 10 minutes, which stained the antigen brown, and then counterstained with Mayer's hematoxylin.

Morphometry

Percentages of positive/total areas with IHC for CK4 and CK13 were estimated using Image J NIH software version 1.40 [24]. For each section of the IULs, the entire epithelium of the lesion was outlined using a freehand selection tool, and the total area of the lesion was measured. Thereafter, the original image was converted into an 8-bit grayscale image containing a blue component, and the lower and upper threshold values were set automatically with the threshold tool. Subsequently, the grayscale image was classified into positive staining areas and background, and the positive staining areas were measured. Finally, the ratios of CK4- and CK13-positive staining for each lesion were obtained as the percentage of the stained area relative to the total area (Fig. 1). According to the morphometric results, IULs were scored as follows: score 1 (preserved: 70% or more), score 2 (intermediate: at least 30% and less than 70%), and score 3 (loss of expression: less than 30%).

MIB-1 labeling index

In all IULs and control samples, the MIB-1 labeling index was obtained as follows: Cell counts were obtained at 400 × magnification using a 10 × 10 eyepiece graticule with conventional light microscope, according to the methods of Dissanayake et al. [25]. The count of cell nuclei was made in three or more consecutive fields by moving the slide, until at least 500 nuclei were scored. Mean MIB-1 labeling index for each sample was estimated as the percentage of immunoreactive nuclei among the total number of nuclei counted.

p53 overexpression

p53 overexpression for each sample was estimated when intense brownish nuclear staining was found in more than 10% of all epithelial cells, including some that spread above the basal cell layer. Weak and scattered staining limited to the basal cell layer was considered negative [26].

Statistical analysis

The chi-square test was used to evaluate the differences in frequency of CK scores among histologic categories, and in status of p53 overexpression among CK scores or histologic categories. Analysis of variance was used to analyze the results of CK-positive ratios and the MIB-1 labeling index. In the correlation analysis of the positive ratio between CK4 and CK13, Pearson's correlation coefficient test was used. In the correlation analysis between CK scores and histologic categories, Spearman's rank correlation coefficient test was used. Differences with p values less than 0.05 were considered to be statistically significant. StatView software (version 5.0; SAS Institute, Cary, NC) was used for the statistical analysis.

Results

Histologic classification and grading of IULs

Representative micrographs of IULs in the study are shown in Figure 2. Of the 154 IULs, 66 (43%) were of HG-IN, 64 (41%) were of LG-IN, and 24 (16%) were of NNL.

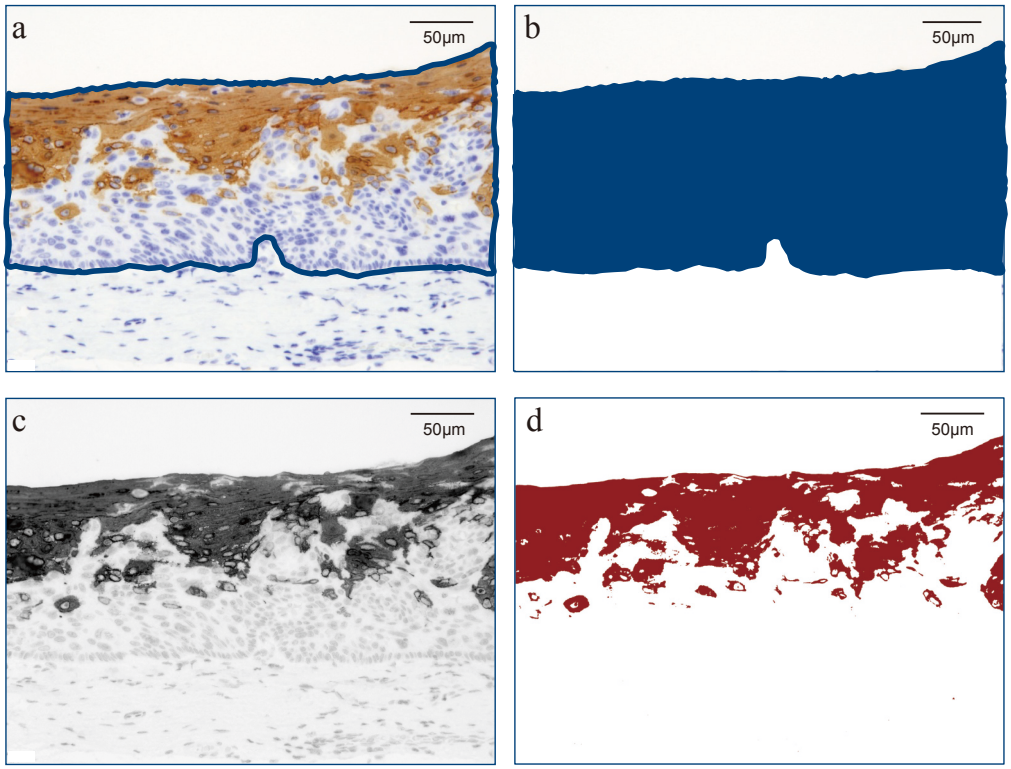

Figure 1 : Morphometric analysis of cytokeratin immunohistochemistry by Image J NIH software version 1.40. The entire epithelium of the lesion is outlined with a blue line (a). The total area of the lesion (shown as blue area) is measured (6,485,655 pixels in this picture) (b). The original image is converted into an 8-bit grayscale image (c). The area of the image exceeding the signal threshold (shown as red area) is considered the positive area of the lesion (2,641,758 pixels in this picture) (d). A ratio of signal-positive to total area is obtained as the percentage of the red area to that of the blue area (40.7% in this lesion).

Decreased expression of CK4 and CK13

Representative micrographs of CK4 and CK13 IHC in the NEE are shown in Figure 3. The CK4-positive staining always showed a consistent and distinct pattern throughout the epithelium, starting from the third or fourth layer of epithelial cells and reaching into the superficial cell layers, but was never detected in the basal cell layer or the suprabasal layers. The CK13-positive cells began from the second layer of epithelial cells and reached into the superficial cell layers, but were never detected in the basal cell layer. In IULs, CK4 and CK13-positive cells were decreased to a varying extent and distribution (Figs. 4 and 5).

Based on the morphometric results, the mean ratio of CK4- and CK13-positive staining were 33% and 48% in HG-IN, 55% and 69% in LG-IN, 73% and 78% in NNL, and 91% and 95% in NEE, respectively (Fig. 6). For both CK4 and CK13, the ratios of positive staining were significantly different between LG-IN and HG-IN, between neoplastic lesion and NNL, and between IUL and NEE, (Table 2; $p < 0.005$). The ratio decreased according to the degree of atypia. Further, a significant positive correlation was observed between the ratios of CK4- and CK13-positive staining (Fig. 7; correlation coefficient = 0.642, $p < 0.0001$).

The results of CK scoring in each category of lesions are shown in Table 3. A significant correlation was also found between CK scores and the degree of atypia, in both CK4 and CK13 ($p < 0.0001$). The 29 lesions with a score of 1 for both CK4 and CK13 included 16 LG-INs and 13 NNLs. The 16 lesions with a score of 3 for both CK4 and CK13 included 15 HG-INs and 1 LG-IN.

Correlation between CK expression and other markers

p53 overexpression was detected in 47/66 (71%) HG-INs, 32/64 (50%) LG-INs, 13/24 (54%) NNLs, and 2/154 (1%) NEEs (Table 4). A significant difference in the frequency occurred between HG-IN and LG-IN ($p < 0.05$), and between IUL and NEE ($p < 0.0001$). Although

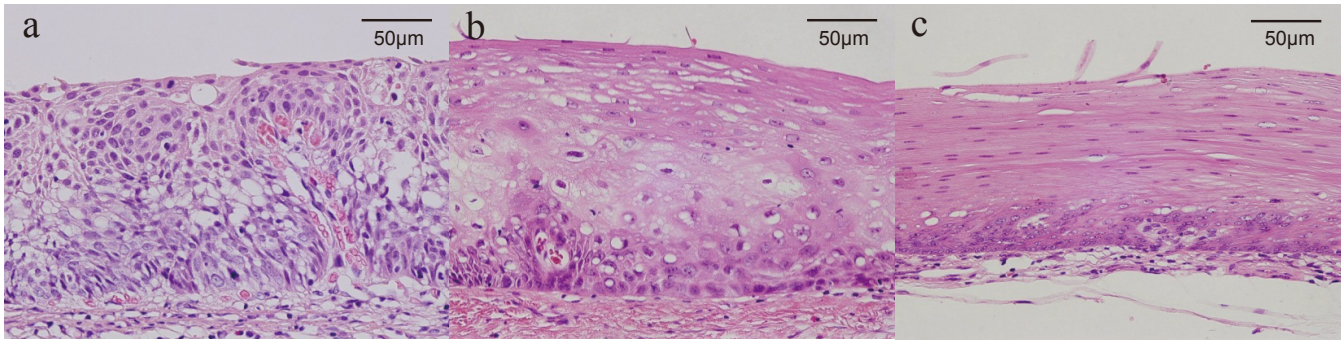

Figure 2 : Histologic classification of iodine-unstained lesions (IULs). Representative histologic micrographs of high-grade intraepithelial neoplasias (HG-IN, a), low-grade intraepithelial neoplasias (LG-IN, b), and non-neoplastic lesions (NNL, c) are shown. In the HG-IN, the abnormal cells also distribute in all of the layers and exhibit a greater degree of atypia (a). In LG-IN, the abnormalities are often confined to the lower half of the epithelium (b). IULs with no neoplastic lesion according to the criteria described above were classified as NNLs (c).

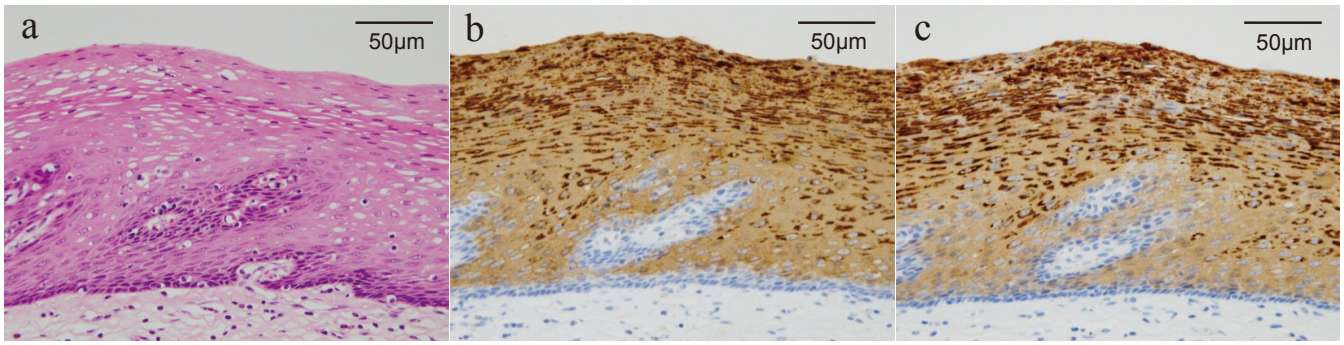

Figure 3 : Cytokeratin 4 and 13 (CK4 and CK13) expression in normal squamous epithelium of the esophagus. Immunostaining patterns of CK4 (center) and CK13 (right) in normal esophageal epithelium are shown together with hematoxylin–eosin staining of each semiserial section (left). CK4-positive staining always showed a consistent and distinct pattern throughout the epithelium, distributing from the third to fourth layer of epithelial cells up to the superficial cell layers, but was never detected in the basal cell layer or the suprabasal layers. CK13-positive cells began from the second layer of epithelial cells and reached into the superficial cell layers, but were never detected in the basal cell layer.

Table 2. CK4- and CK13-positive staining ratios in each histologic category and NEE

	Total number of IULs	CK4-positive staining ratio (mean±SD, %)[a]	CK13-positive staining ratio (mean±SD, %)[b]
HG-IN	66	32.5±21.1	48.0±22.7
LG-IN	64	55.4±22.2	69.0±16.9
NNL	24	73.4±15.7	78.0±17.1
NEE	154	90.5± 3.1	94.5± 2.1

IUL, iodine-unstained lesion; HG-IN, high-grade intraepithelial neoplasia; LG-IN, low-grade intraepithelial neoplasia; NNL, non-neoplastic lesion; NEE, normal esophageal epithelium; a-b, $p < 0.0001$ (analysis of variance).

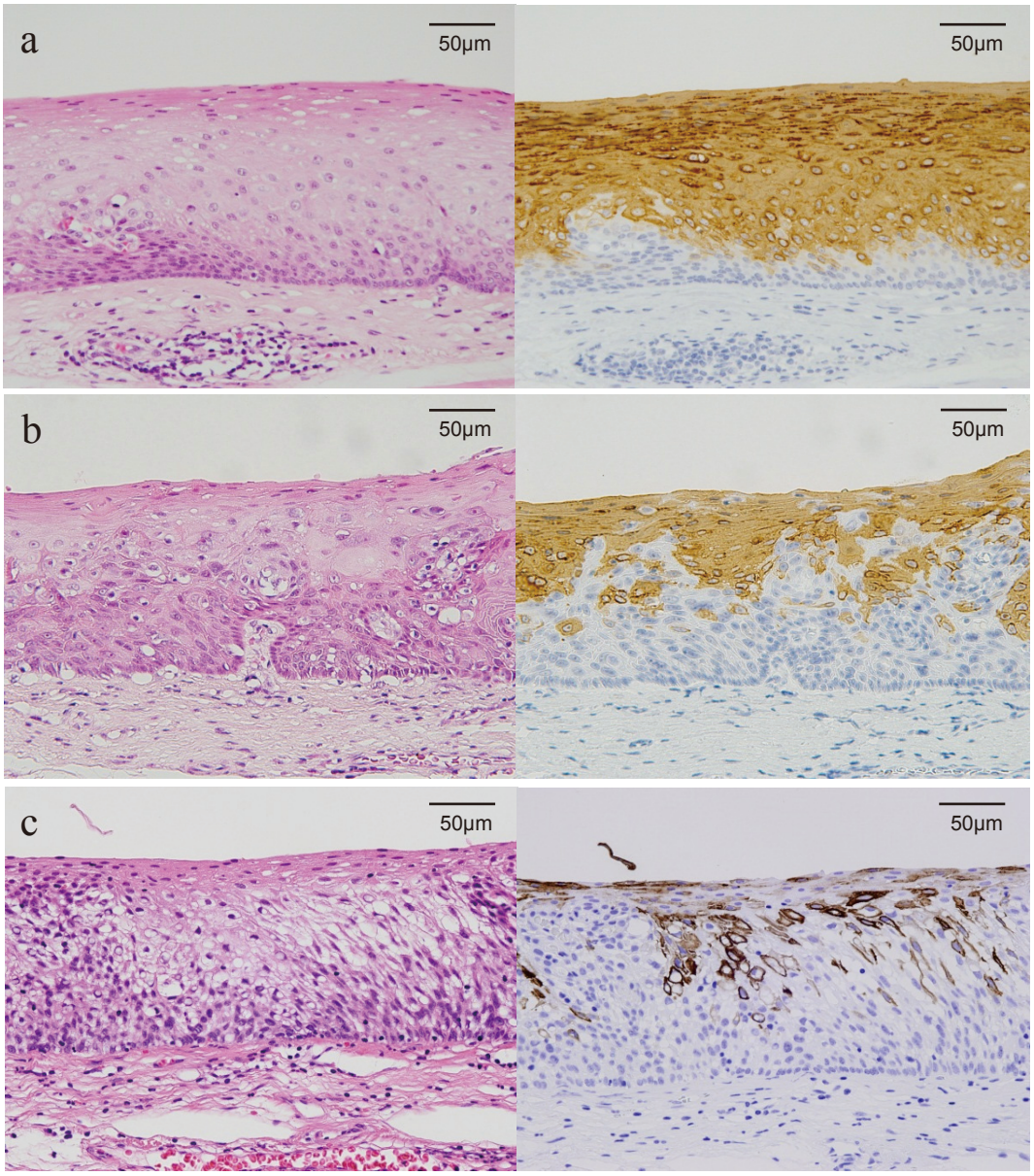

Figure 4 : Cytokeratin 4 (CK4) expression in iodine-unstained lesions (IULs). Representative immunostaining patterns of CK4 in IULs are shown together with hematoxylin–eosin staining of each semiserial section (left). (a) Reduced expression of CK4 was not prominent and was classified with a score of 1 (CK4-positive ratio: 81.8% by morphometry). This lesion was graded as non-neoplastic lesion. (b) The number of CK4-negative cells moderately increased in the lesion. This lesion was graded as high-grade intraepithelial neoplasia and was classified with a score of 2 (CK4-positive ratio: 43.5% by morphometry). (c) Reduced expression of CK4 was prominent and classified with a score of 3 (CK4-positive ratio: 12.5% by morphometry). This lesion was graded as high-grade intraepithelial neoplasia.

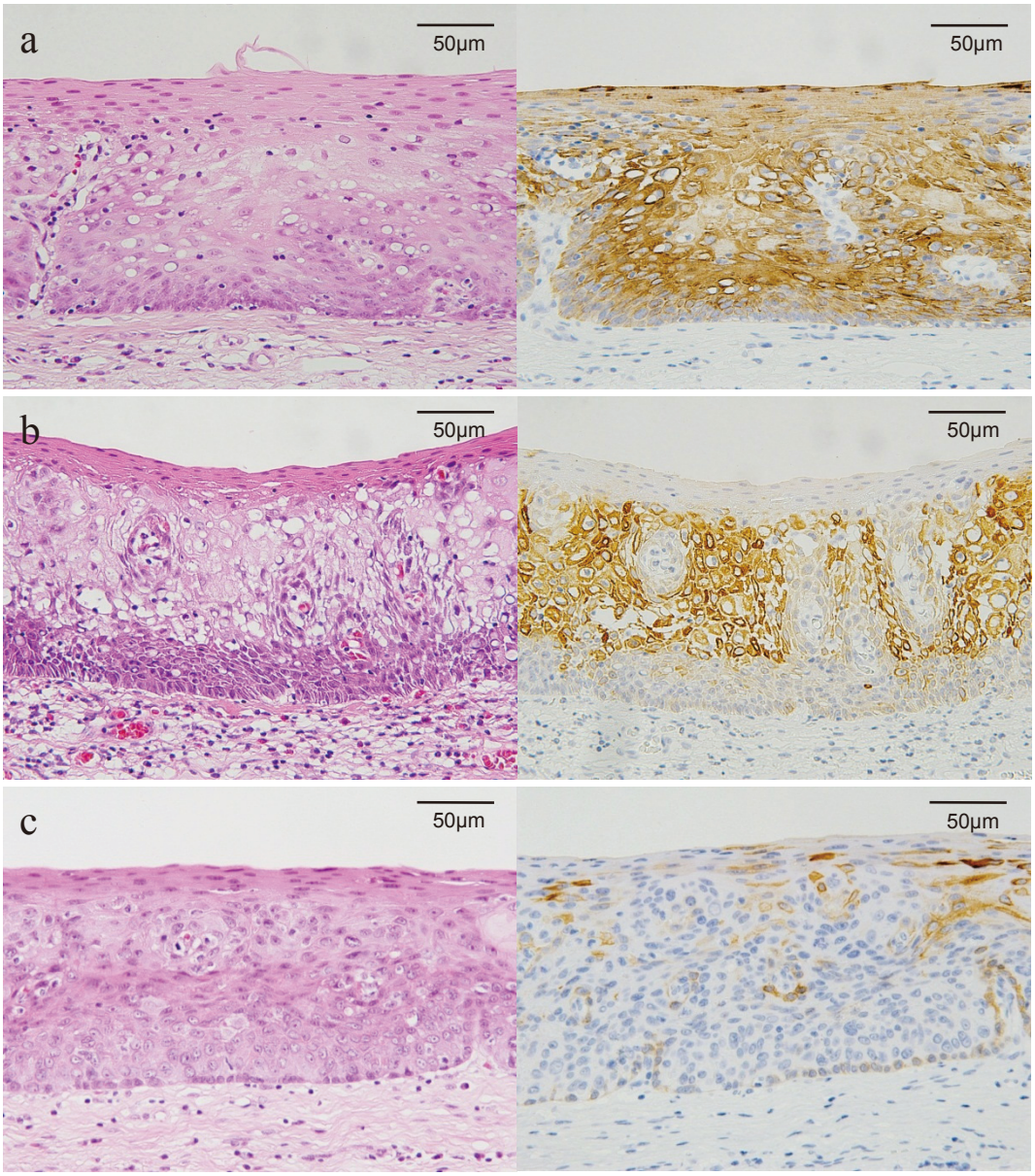

Figure 5 : Cytokeratin 13 (CK13) expression in iodine-unstained lesions (IULs). Representative immunostaining patterns of CK13 in IULs are shown together with hematoxylin–eosin staining of each semiserial section (left). (a) Reduced expression of CK13 was not prominent and classified with a score of 1 (CK13-positive ratio: 77.8% by morphometry). This lesion was graded as low-grade intraepithelial neoplasia. (b) The number of CK13-negative cells moderately increased in the lesion. This lesion was graded as low-grade intraepithelial neoplasia and classified with a score of 2 (CK13-positive ratio: 58.1% by morphometry). (c) Reduced expression of CK13 was prominent and classified with a score of 3 (CK13-positive ratio: 17.9% by morphometry). This lesion was graded as high-grade intraepithelial neoplasia.

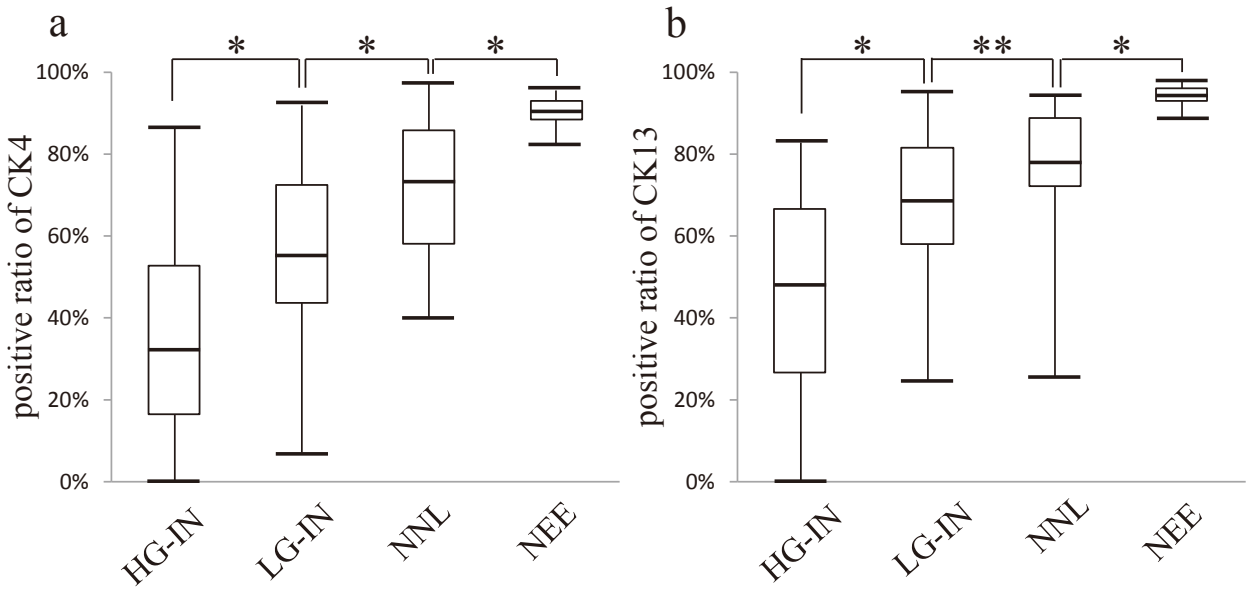

Figure 6 : Morphometric analysis of cytokeratin immunohistochemistry. The results of CK4- (a) and CK13-positive ratio (b) in high-grade intraepithelial neoplasia (HG-IN), low-grade intraepithelial neoplasia (LG-IN), non-neoplastic lesion (NNL), and normal esophageal epithelium (NEE) are shown with box and whisker plots. Bars indicate maximum value and minimum value, and boxes indicate 75%-tile and 25%-tile with lines of median values inside the boxes. The ratios of CK4- and CK13-positive staining were significantly different between HG-IN and LG-IN, between neoplastic and non-neoplastic lesions, and between iodine-unstained lesions and NEE (* $p < 0.0001$, ** $p = 0.0027$).

Table 3. Relationship between CK scores and histologic grade

CK4 score	CK13 score	Total number of IULs	Number of HG-IN	Number of LG-IN	Number of NNL	Spearman's rank correlation coefficient test
1	-	34	1	18	15	
2	-	77	31	37	9	r = 0.61, $p < 0.0001$
3	-	43	34	9	0	
-	1	64	12	33	19	
-	2	69	37	28	4	r = 0.56, $p < 0.0001$
-	3	21	17	3	1	
1	1	29	0	16	13	
1	2	4	1	1	2	
1	3	1	0	1	0	
2	1	31	11	14	6	
2	2	42	18	22	2	
2	3	4	2	1	1	
3	1	4	1	3	0	
3	2	23	18	5	0	
3	3	16	15	1	0	

IUL, iodine-unstained lesion; HG-IN, high-grade intraepithelial neoplasia; LG-IN, low-grade intraepithelial neoplasia; NNL, non-neoplastic lesion; NEE, normal esophageal epithelium; r, corFrelation coefficient.

Table 4. p53 overexpression status and MIB-1 labeling index in each histologic category and NEE

	Total number of IULs	Number of IULs with p53 overexpression (%)	MIB-1 labeling index (mean±SD, %)[c]
HG-IN	66	47 (71.2) [a]	45.3±15.1
LG-IN	64	32 (50.0) [a]	26.1±11.7
NNL	24	13 (54.2) [b]	11.0± 6.8
NEE	154	2 (1.3) [b]	6.5± 2.6

IUL, iodine-unstained lesion; HG-IN, high-grade intraepithelial neoplasia; LG-IN, low-grade intraepithelial neoplasia; NNL, non-neoplastic lesion; NEE, normal esophageal epithelium; a, $p < 0.05$; b, $p < 0.0001$; c, $p < 0.0001$

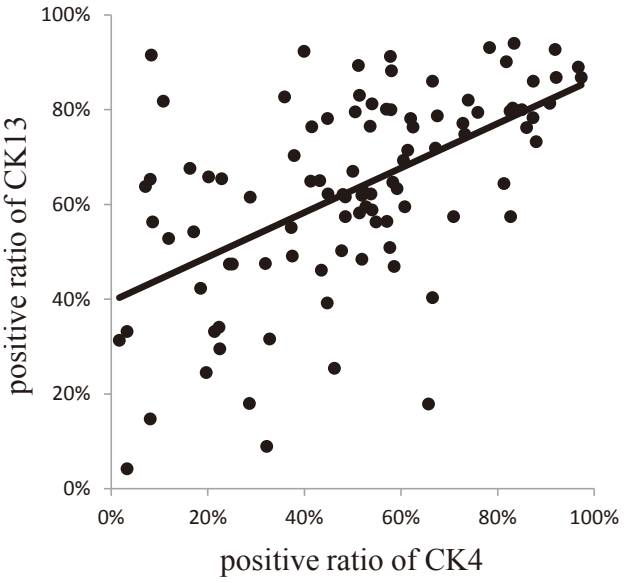

Figure 7. The correlation between the ratios of CK4- and CK13-positive staining. The correlation between the CK4- and CK13-positive ratios of the all IULs is shown. The x- and y- axes show the percentage of the CK4- and CK13-positive area relative to the total area of each lesion, respectively. A significant positive correlation was observed (correlation coefficient = 0.642, $p < 0.0001$).

the status of p53 overexpression was not correlated with the CK expression scores, the MIB-1 labeling index significantly increased with an increase in the CK4 and CK13 scores (Table 5). Mean MIB-1 labeling index was 45% in HG-IN, 26% in LG-IN, 11% in NNL, and 7% in NEE. The difference of the mean index was significant among HG-IN, LG-IN, and NNL ($p < 0.0001$), but not between NNL and NEE (Table 4).

Discussion

CKs are the most diverse intermediate filament family. Every CK is made up of an equal mixture of type I (acidic) and type II (neutral/basic) keratin chains; these form heterodimers, two of which then join to form a tetrameric subunit. In the non-keratinizing squamous epithelium, neutral or basic CK4 and acidic CK13 form a heterodimer and exist as tetramers [27].

Normal esophageal epithelium is multilayered, and is largely composed of non-keratinizing squamous cells. These cells change their appearance from one layer to the next. Those in the innermost layer, attached to the underlying basal lamina, are termed basal cells, and never express both CK4 and CK13. Above the basal cells are one or two cell layers of parabasal (suprabasal) cells, which usually have proliferative activity and express CK13, but not CK4. The next larger prickle cells, whose numerous desmosomes—each a site of anchorage for thick tufts of keratin filaments—are just visible in the light microscope as tiny prickles (so-called intercellular bridges) around the cell surface. Beyond the prickle cells lies the superficial layer (functional layer), consisting of flattened cells whose boundaries are difficult to detect with a light microscope. The prickle cells and superficial cells express both CK4 and CK13. Therefore, dimerization of CK4 and CK13 is considered to occur in the transition from the parabasal layer to the prickle cell layer.

The expression of CK4 and CK13 is regulated with cellular differentiation, and CK disruption results in an impairment of the normal differentiation sequence [28]. Therefore, the IULs in the present study may be regarded as an abnormal condition of cellular differentiation. Although the biologic implications and molecular mechanisms of CK abnormalities have not yet been clarified, understanding the role of cytoskeletal components in the determination of cell differentiation might be important in the context of esophageal cancer progression.

Recently, a multistep model of carcinogenesis of the esophagus as well as other organs was developed [2]. An epidemiologic follow-up study by Dawsey et al. [29]

Table 5. Relationship between CK scores and MIB-1 labeling index, or p53 overexpression

CK4 score	CK13 score	Total number of IULs	MIB-1 labeling index (mean±SD, %)	*p* value[a]	Number of Lesions with p53 overexpression	*p* value[b]
1	-	34	19.8±10.9		17	
2	-	77	31.3±15.8	< 0.0001	47	0.38
3	-	43	42.8±19.3		28	
-	1	64	23.3±11.5		37	
-	2	69	37.5±18.9	< 0.0001	42	0.91
-	3	21	40.1±19.3		13	
1	1	29	19.5±10.4		15	
1	2	4	25.4±12.3		2	
1	3	1	7.8±0.0		0	
2	1	31	27.1±11.1		19	
2	2	42	34.6±17.0		25	
2	3	4	29.8±23.4		3	
3	1	4	22.0±12.4		3	
3	2	23	45.1±20.5		15	
3	3	16	44.7±15.5		10	

IUL, iodine-unstained lesion; [a] analysis of variance, [b] Chi-square test.

suggests an increased risk for the subsequent development of esophageal invasive squamous cell carcinoma for patients with low-grade dysplasia (relative risk, RR: 2.2), moderate-grade dysplasia (RR: 15.8), high-grade dysplasia (RR: 72.6), and carcinoma *in situ* (RR: 62.5). Therefore, it is very important to evaluate the histologic grade, which indicates the risk for progression to invasive carcinoma. In the present study, the degree of the reduction of CK-positive cells correlated to the histologic grade of esophageal intraepithelial neoplasia. While the present criteria for histologic grading is relatively subjective, IHC for CKs could be helpful for developing an objective grading method, as demonstrated in the present study.

The MIB-1 labeling index in IULs also correlated with the histologic grade. Kushner et al. [30] reported that in an IHC study of the biopsy specimens from the epithelial dysplasia of the mouth, the mean MIB-1 labeling index of the dysplasia was significantly higher than that of normal epithelium and the MIB-1 labeling index increased with an increase in the grade of dysplasia. Therefore, the MIB-1 labeling index as well as the CK4 and CK13 expression scores in esophageal mucosal lesions with iodine-negative staining may indicate the risk for progression to invasive carcinoma.

Interestingly, p53 overexpression was detected in 54% of NNLs in the study. This finding suggests that NNLs are precancerous lesions in an extremely-early stage of esophageal carcinogenesis, despite the fact that these lesions are difficult to diagnose as neoplasia by conventional histology. Kaneko et al. [31] suggested that some IULs with non-dysplastic epithelium, referred to as NNLs in the present study, represent the earliest state of esophageal squamous cell carcinoma by p53 mutation analysis. Further, Fagundes et al. [25] reported that in high-risk groups of heavy smokers and alcohol drinkers, p53 overexpression is observed even in iodine-stained esophageal mucosa with a histologically normal appearance. Thus, evaluation of p53 overexpression is useful for detecting initial neoplastic changes, but may not be an indicator of the risk for progression to invasive carcinoma.

Although evaluating the CK-positive ratio by morphometric methods using Image J NIH software is objective, it is too complex and time-consuming for practical diagnosis. Thus, for practical use, we classified IULs with scores ranging from 1 to 3 according to the percentages of the CK-positive cells. Using this scoring method, a score of 3 for both CK4 and CK13 was strongly suggestive of HG-IN. A score of 1 for both CK4 and CK13 was suggestive for the lower-risk lesions (LG-IN and NNL), except for HG-IN. Based on our results, when it is difficult to define histologic grade by conventional histology such as hematoxylin–eosin or periodic acid-Schiff staining, IHC scoring for CK4 and CK13 is helpful toward achieving a more accurate diagnosis.

In conclusion, IHC for the expression of CK4 and

CK13 together with the MIB-1 labeling index proved to be an objective and useful method for histopathologic evaluation of the risk for progression to invasive carcinoma in IULs, resulting in adequate therapy.

Conflict of interest

The authors declare that they have no conflicts of interest. This study was approved by conflict of interest committee of Tokyo Medical and Dental University.

Acknowledgement

The authors thank Dr. Kei Sakamoto, assistant professor of the Tokyo Medical and Dental University, and Dr. Toichiro Takizawa, former professor of Tokyo Medical and Dental University, for providing idea of this study and sincere encouragement.

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