Original

**An Immunohistological Study on Intraepithelial
Lesions in Esophagus**

— Expression of GLUT-1, Cytokeratin, and p53, with Ki-67 Staining —

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Abstract : Esophageal epithelial lesions are important features with respect to the early diagnosis of esophageal cancer. Such lesions include inflammatory reactive lesions (inflammatory reactions, IR) and neoplastic lesions. However, while neoplastic lesions are also classified from low-grade intraepithelial neoplasia (LN) to high-grade intraepithelial neoplasia (HN), it is often difficult to differentiate esophageal epithelial lesions in a biopsy. Therefore, this study analyzed cases of endoscopic mucosal resection (EMR) and dissection by immunostaining for p53, MIB-1, glucose transporter-1 (GLUT-1), and cytokeratin (CK) in addition to cytohistological grading to establish lesions trends for classification. This study included 50 lesions of EMR performed at our hospital from 2004 to 2007, as well as 31 cases who underwent a pathological autopsy at our hospital during the period between 2006 and 2007. Tissue diagnoses by HE staining classified the specimens into five groups: normal, IR, LN, HN, and squamous cell cancer (SCC). This study undertook further comparison by immunostaining for p53, Ki-67 staining, GLUT-1 and CKs 17 and 14. All immunostaining revealed a significant difference between IR and LN grade of lesions. Specifically, GLUT-1 staining revealed a significant difference between LN and HN lesions, while CK17 staining differentiated significantly between HN and SCC. The findings indicated that pathological diagnoses of esophageal lesions should include GLUT-1, CK17, and CK14 immunostaining to differentiate LN and HN lesions.

Key words : esophageal epithelial lesions, intraepithelial neoplasia, glucose transporter-1, cytokeratin

Introduction

Esophageal epithelial lesions are important early indicators of esophageal cancer. Esophageal epithelial lesions include inflammatory reactive (IR) lesions and neoplastic lesions.

Dysplasia was used to describe intraepithelial tumors in the past, but the term intraepithelial neoplasia was introduced in the WHO classification of 2000¹⁾. Neoplastic lesions are also differentiated as low-grade intraepithelial neoplasia (LN) or high-grade intraepithelial neoplasia (HN) including cancer in situ by the Japanese Classification of Esophageal Cancer (10th Edition) in Japan²⁾, and different treatments are recommended for LN and HN lesions, necessitating strict differentiation. However, it is often difficult to differentiate these lesions in a biopsy. In addition, recent advances in diagnostic techniques using magnifying endoscopes and endoscopes³⁻⁵⁾ has also defined ultra-microcarcinomas of 1 mm⁶⁾. These factors and that esophageal cancer is a possible causal factor in elderly cases of dysphagia make early detection even more important.

This study examined the use of immunostaining for p53, Ki-67, glucose transporter-1 (GLUT-1), and cytokeratin (CK) in addition to cytohistological grading to compare and distinguish esophageal lesions.

Materials and Methods

Subjects

The study analyzed 50 lesions in 49 cases (38 males and 12 females, average age: 64.4 ± 8.9 years) diagnosed as atypical epithelium or squamous cell cancer (SCC) of the esophageal mucosa and who underwent endoscopic mucosal resection (hereinafter referred to as "EMR") at our hospital from 2004 to 2007, as well as 31 control cases (20 males and 11 females, average age: 69.7 ± 12.6 years) with no esophageal lesions who underwent a pathological autopsy at our hospital from 2006 to 2007.

Histological study

Specimens were fixed in formalin and paraffin-embedded, before preparation of sections for hematoxylin and eosin (HE) staining and microscopy. With reference to the Japanese Classification of Esophageal Cancer (10th Edition)²⁾ and various research papers published in Japan⁷⁻⁹⁾, the specimens were diagnosed as normal, IR, LN, HN, or SCC, based on agreement between two pathologists regarding cellular atypia and structural atypia (Fig. 1).

Immunohistochemistry

Immunostaining was performed to localize p53, Ki-67, GLUT-1, CK17, and CK14 expression. Briefly, sections were deparaffinized and washed, before heat-treatment activation with EDTA (pH 8.5) and incubation with primary antibody (DAKO, Denmark 50-fold dilution, reaction time: 32 minutes). The sections were then incubated in biotin marker Ig (Ventana Germany) as a secondary antibody and DAB was used to visualize the immunoreaction.

The primary antibodies used were as follows: anti-p53 (DO-7 monoclonal antibody) and anti-Ki-67 (MIB-1 monoclonal antibody) from Dako and used at 1: 50 dilution; anti-GLUT-1 (polyclonal) from Dako and used at 1: 100; anti-CK14 (polyclonal) from Progen

Table 1. Immunological study

	Maker	Attenuation magnification	origin animal	Production country
GLUT-1 (polyclonal)	Dako	× 100	Rabbit	Denmark
P53 (DO-7)	Dako	× 50	Mouse	Denmark
Ki-67 (MIB-1)	Dako	× 50	Mouse	Denmark
CK14 (Polyclonal)	PROGEN	× 100	Pig	Australia
CK17 (E3)	Dako	× 25	Mouse	Denmark

(Australia) and used at 1 : 100 ; and, anti-CK17 (E3 monoclonal) from Dako and used at 1 : 25.

Staining was performed using the Ventana HX System Benchmark automatic staining system (Table 1).

Manifestation characteristics

We classified p53 and Ki-67 staining by comparison between stainability of p53 and Ki-67 based on Watanabe *et al*¹⁰⁾. Cases in which Ki-67-positive cells were seen in the more upper layers compared to p53-positive cells, as well as cases in which positive cells were only seen in three rows or lower from the basal side in p53 staining, were classified as type 1, while cases in which p53-positive cells were seen in 4 to 6 layers from the basal side were classified as type 2, with 7 to 9 layers classified as type 3, and 10 layers or more as type 4 (Fig. 2).

In GLUT-1, cases that were negative or weakly positive in a few layers of the basal portion were classified as type 1, cases that were strongly positive in a few layers of the basal portion as type 2, cases that were strongly positive in all layers as type 3, and cases that were strongly positive in the surface part but negative in the basal side as type 4 (Fig. 3).

In CK14 staining, cases that were strongly positive in 1 or 2 layers of the basal side were classified as type 1, cases that were strongly positive in a few layers of the basal side as type 2, cases that were positive in all layers as type 3, and cases that were strongly positive in the surface part but negative in the basal side as type 4 (Fig. 4).

In CK17, negative cases were classified as type 1, cases in which stainability was discontinuous and partially observed were classified as type 2, cases with continuous staining but not in all layers as type 3, and cases with staining in all layers were classified as type 4 (Fig. 5).

Evaluation method

Each classification was scored (type 1 is 1 point, type 2 is 2 point, type 3 is 3 points, and type 4 is 4 points). Data were compared using an $m \times n$ table χ^2 test, with $P < 0.05$ considered to indicate a significant difference.

Fig. 1. Examples of lesions classified by HE staining

- a. Normal : Only slight inflammatory cell infiltration is observed within the epithelium and the basal cells are finely aligned.
- b. IR : The basal cells are slightly out of alignment but no nuclear atypicity or front formation is observed, and inflammatory cell infiltration is observed within the epithelium.
- c. LN : Front formation is observed along with nuclear atypicity and increased cell density, but the nuclear atypicity is mild.
- d. HN : Compared to LN, the nuclear atypicity is strong and the cell density has increased, but no interstitial infiltration is involved.
- e. SCC : Obvious interstitial infiltration is observed.

Fig. 2. Examples of lesions classified by p53 and Ki-67 staining (upper : p53 ; lower : Ki-67)

- a. type 1 (1 point) : Ki-67-positive cells are observed in the more upper layers compared to p53-positive cells, or positive cells are observed in only three rows or less from the basal side with p53 staining.
- b. type 2 (2 points) : The stainability of p53 staining is stronger than that of Ki-67 staining, and positive cells are observed in 4 to 6 rows from the basal side with p53 staining.
- c. type 3 (3 points) : The stainability of p53 staining is stronger than that of Ki-67 staining, and positive cells are observed 7 to 9 rows from the basal side with p53 staining.
- d. type 4 (4 points) : The stainability of p53 staining is stronger than that of Ki-67 staining, and positive cells are observed in 10 rows or more from the basal side with p53 staining.

Fig. 3. Examples of lesions classified by GLUT-1 staining

- a. type 1 (1 point) : Negative or weakly positive in a few layers from the basal side
- b. type 2 (2 points) : Strongly positive in a few layers from the basal side
- c. type 3 (3 points) : Strongly positive in almost all layers (slightly expanding)
- d. type 4 (4 points) : Strongly positive in the surface part but negative on the basal side

Fig. 4. Examples of lesions classified by CK14 staining

- a. type 1 (1 point) : Strongly positive in 1 to 2 layers from the basal side
- b. type 2 (2 points) : Strongly positive in a few layers from the basal side
- c. type 3 (3 points) : Strongly positive in almost all layers
- d. type 4 (4 points) : Strongly positive in the surface part but negative on the basal side

Fig. 5. Examples of lesions classified by CK17 staining

- a. type 1 (1 point) : Negative
- b. type 2 (2 points) : A discontinuous positive appearance is observed.
- c. type 3 (3 points) : A continuous positive appearance is observed.
- d. type 4 (4 points) : Strongly positive in all layers

Results

Diagnoses of the HE-stained specimens for the total of 81 lesions was normal in 22 subjects, IR in 9 subjects, LN in 11 subjects, HN in 19 subjects, and SCC in 20 subjects.

For p53 and Ki-67 staining, the normal group scored 1.00 ± 0.00 point (1 point : 22 subjects), with the IR group at 1.00 ± 0.00 point (1 point : 9 subjects), the LN group at 2.73

Table 2. Frequency of expression of p53 / Ki-67

	1	2	3	4	Ave.
Normal	22	0	0	0	1.00±0.00
IR	9	0	0	0	1.00±0.00
LN	1	3	5	2	2.73±0.90
HN	6	0	2	11	2.95±1.39
SCC	5	0	2	13	3.15±1.31

Fig. 6. Study of each staining result

±0.90 points (1 point: 1 subject; 2 points: 3 subjects; 3 points: 5 subjects; 4 points: 2 subjects), the HN group at 2.95±1.39 points (1 point: 6 subjects; 3 points: 2 subjects; 4 points: 11 subjects), and the SCC group at 3.15±1.31 points (1 point: 5 subjects; 3 points: 2 subjects; 4 points: 13 subjects) (Table 2). Frequency of expression in the LN group was 2 points or more and therefore significantly higher than those in the IR group (Fig. 6-a).

For GLUT-1 staining, the normal group scored 1.00±0.00 point (1 point: 22 subjects), with the IR group at 1.00±0.00 point (1 point: 9 subjects), the LN group at 2.00±0.77

Table 3. Frequency of expression of GLUT-1

	1	2	3	4	Ave.
Normal	22	0	0	0	1.00±0.00
IR	9	0	0	0	1.00±0.00
LN	2	8	0	1	2.00±0.77
HN	1	6	7	5	2.84±0.90
SCC	0	5	8	7	3.10±0.79

Table 4. Frequency of expression of CK14

	1	2	3	4	Ave.
Normal	21	0	1	0	1.09±0.43
IR	8	0	1	0	1.22±0.67
LN	2	2	4	3	2.73±1.10
HN	2	2	9	6	3.00±0.94
SCC	0	0	14	6	3.30±0.47

points (1 point: 2 subjects; 2 points: 8 subjects; 4 points: 1 subject), the HN group at 2.84 ± 0.90 points (1 point: 1 subject; 2 points: 6 subjects; 3 points: 7 subjects; 4 points: 5 subjects), and the SCC group at 3.10 ± 0.79 points (2 points: 5 subjects; 3 points: 8 subjects; 4 points: 7 subjects) (Table 3). Frequency of expression in the LN group was 2 points or more and therefore significantly higher than in the IR group, but significantly lower than in the HN group, in which the frequency of expression was 3 points or more (Fig. 6-b).

For CK14 staining, the normal group scored 1.09 ± 0.43 points (1 point: 21 subjects; 3 points: 1 subject), with the IR group at 1.22 ± 0.67 points (1 point: 8 subjects; 3 points: 1 subject), the LN group at 2.73 ± 1.10 points (1 point: 2 subjects; 2 points: 2 subjects; 3 points: 4 subjects; 4 points: 3 subjects), the HN group at 3.00 ± 0.94 points (1 point: 2 subjects; 2 points: 2 subjects; 3 points: 9 subjects; 4 points: 6 subjects), and the SCC group at 3.30 ± 0.47 points (3 points: 14 subjects; 4 points: 6 subjects) (Table 4). Frequency of expression in the LN group was 2 points or more and therefore significantly higher than in the IR group (Fig. 6-c).

For CK17 staining, the normal group scored 1.00 ± 0.00 point (1 point: 22 subjects), with the IR group at 1.00 ± 0.00 point (1 point: 9 subjects), the LN group at 1.91 ± 0.83 (1 point: 4 subjects; 2 points: 4 subjects; 3 points: 3 subjects), the HN group at 2.21 ± 0.85 (1 point: 4 subjects; 2 points: 8 subjects; 3 points: 6 subjects; 4 points: 1 subject), and the SCC group at 3.15 ± 0.75 (2 points: 4 subjects; 3 points: 9 subjects; 4 points: 7 subjects) (Table 5). Frequency of expression in the LN group was 2 points or more and therefore significantly higher than in the IR group, while in the SCC group, the frequency of appearance at 3 points or more was significantly higher compared to the HN group (Fig. 6-d).

Table 5. Frequency of expression of CK17

	1	2	3	4	Ave.
Normal	22	0	0	0	1.00±0.00
IR	9	0	0	0	1.00±0.00
LN	4	4	3	0	1.91±0.83
HN	4	8	6	1	2.21±0.85
SCC	0	4	9	7	3.15±0.75

In a comparison of elderly (65 years of age or older) compared to younger patients (<65 years old), 39 subjects (normal: 8 subjects, IR: 3 subjects, LN: 7 subjects, HN: 10 subjects, SCC: 11 subjects) were in the former group, while 42 subjects (normal: 14 subjects, IR: 6 subjects, LN: 4 subjects, HN: 9 subjects, SCC: 9 subjects) were <65 years old. Immunostaining showed that the frequency of expression of type 3 and type 4 was significantly high in the elderly cases of the HN group compared to younger cases, but no other significant differences were observed.

Discussion

According to the WHO classification, lesions localized in half or less of the basal side of the squamous epithelium are classified as LN, whereas those localized in half or more are classified as HN¹⁾. However, in cases where the esophageal epithelium shows epithalaxia or an eroded surface layer, it is difficult to differentiate based on only the height of all of the layers of the esophageal epithelium. Therefore, the Japanese Classification of Esophageal Cancer (Tenth Edition)²⁾ in Japan recommends that cellular atypia and structural atypia are considered and many research papers in Japan have cited these features for differentiating esophageal epithelial lesions. However, in reality it is not always easy to differentiate these lesions, and there is no consensus among pathologists in Japan⁷⁻⁹⁾. From the perspective of treatment strategy, however, it is very important to differentiate these lesions. For both IR and LN, treatment is not necessary and follow-up is sufficient. However, LN lesions can develop into HN, which might require demucosation, so stricter follow-up is necessary. Recent advances in endoscopic diagnostic techniques such as magnifying endoscopy³⁻⁵⁾ have allowed ultra-microcarcinomas of 1 mm to be detected⁶⁾, and prognoses have thereby been changed to a large degree by the early diagnoses of esophageal cancer.

Immunostaining for p53 and Ki-67 are commonly used to characterize esophageal epithelial lesions in Japan. Watanabe, *et al*¹⁰⁾ described that in normal or inflammatory reactive epithelia, p53-positive cells might be found in all three layers from the basal layer, with positive cells in higher layers indicating a high possibility of neoplastic lesion, as does a p53 or Ki-67 marker index of 100% or higher. However, there are no reports on the value of differentiating among LN, HN, and SCC. In the present study, LN lesions showed a significantly higher rate of p53-positive cells than the IR group, but there were no obvious differ-

ences in distribution between the LN group and the HN group or between the HN group and the SCC group. This was attributed to the fact that the p53-positive rate in cases of progressive esophageal cancer is approximately 70%¹¹⁾ and that there were few negative cases in the HN or SCC group in the present study. It was also believed that the distributions became similar because positive cells were observed in ten layers or more in many of the positive cases. Therefore, it is believed that a p53 or Ki-67 marker index is useful to differentiate neoplastic from nonneoplastic cases, but further study is required to differentiate between LN and HN.

GLUT-1 is part of the glucose transporter family of genetic factors that includes GLUT-1 to 5 and 7. GLUT-1 functions as a glucose replacer/regulator that efficiently conducts glucose transport between the inside and outside of a cell membrane¹²⁻¹⁴⁾. In the present study, the IR group showed a type 1 staining pattern, but LN lesions were type 2 or higher. It is believed that type 2 or higher staining pattern indicates a high possibility of a lesion of LN or higher. In the HN group, lesions of type 3 and type 4 were observed at high rates, and significant differences were observed in the rates of type 1 + type 2 and type 3 + type 4 between the LN group and the HN group ($p=0.004$). Based on this fact, it is believed that when a strongly positive picture in all layers or negativity on the basal side is observed with GLUT-1 staining, there is a high possibility of a lesion of HN or higher.

GLUT-1 expression is also a marker of tumor hypoxia, tends to be strongly expressed in the center of cancer tissues^{15,16)}, is mostly expressed on tumor cells in a hypoxic state, and is observed in a site distant from the blood vessels or in tumor cells around a necrotic focus. Therefore, a pattern in which positivity is exhibited in the tumor cells makes sense, but there are no reports of the basal side becoming negative or any mechanisms proposed. Negativity on the basal side is frequently observed, particularly in high-grade tumors, as is the proliferation of blood vessels around the tumors, indicating the involvement of such negativity. Moreover, GLUT-1 expression in association with cell proliferation, glucose activity, and metabolism has also been indicated, and many cases of positivity in the high-grade atypical epithelium of the uterine cervix, but negativity in the cancer pearl and the significantly cornified cells have been reported^{17,18)}. Therefore, it is possible that the basal side exhibits negativity particularly in tumors with a strong tendency toward cornification.

CK is an intermediate filament of 40 to 68 kDa with 20 subunits (CK1 to 20) that differ in molecular weight and isoelectric point by two-dimensional electrophoresis^{19,20)}. There are two major types of keratin pairs present in the esophageal squamous epithelium: at 51 kDa and 59 kDa, and at 50 kDa and 58 kDa, while cancer cells and cultured cells could express other keratin types that do not appear in a normal epithelium, such as 46 kDa, 48 kDa, and 56 kDa forms. These variations indicate that keratin could distinguish esophageal squamous cell cancer from a normal epithelium^{21,22)}.

CK14 is equivalent to high-molecular keratin with a molecular weight of 50 kDa. It is expressed mainly in basal cells of a normal esophageal stratified squamous epithelium, but

not in most cases of squamous cell cancer²³⁾. In the normal and IR groups in the present study, most of the subjects showed strong CK14 positivity only in 1 to 2 rows of the basal side, in neoplastic lesions with LN or higher, and in lesions of type 2 or higher. It should be compatible that the positive cells which appear in many layers during neoplastic changes may be caused by the proliferation of the basal cells. Moreover, lesions that indicate a pattern such as type 4, in which the basal side exhibits negativity, were occasionally observed, indicating proliferation without the formation of normal keratin. However, no significant differences were observed in distribution between the LN group and the HN group or the HN group and the SCC group, so the value of CK14 for differentiating grades of neoplastic lesions was not apparent.

CK17 is equivalent to low-molecular keratin with a molecular weight of 46 kDa and exhibits negativity both in the basal layer and the surface layer of a normal stratified squamous epithelium²⁴⁾. In the normal and IR groups in this study, all of the subjects were CK17-negative, while positive cells were observed in the neoplastic lesions of LN or higher. Between the LN group and the HN group, similar distributions were observed and no significant differences were observed, but the SCC group lesions exhibited a higher rate of type 4 staining and no negative specimens. As the grade became higher, the stainability increased, indicating that in tumors with a strong tendency toward infiltration, there was a possibility that keratin with a molecular weight that does not appear in a normal esophageal epithelium would appear at a high rate. Indeed, Mituo²⁵⁾ described a keratin with a different molecular weight from the standard form in esophageal squamous cell carcinoma.

In elderly cases, surgery involves high risks and chemoradiation therapy is often selected for progressive cancer. However, if endoscopic treatment is possible as a result of early detection, there is a higher chance of complete cure. Therefore, treatment due to early detection is important, particularly in elderly patients, to affect the prognosis as well as the quality of life. In the present study, few significant differences were observed in stainability between the elderly and nonelderly cases, but such studies should be continued because the number of elderly people undergoing endoscopy will increase due to the aging population, and the number of cases that will be detected at an early stage will increase with more accurate pathological diagnoses.

Conclusion

Diagnosis of a lesion is important for cases of LN and HN, because the treatment policies for each condition differ. According to the results of the present study, GLUT-1, CK17, and CK14 immunostaining are useful additions in diagnosing an esophageal lesion. GLUT-1 staining was particularly valuable in differentiating between LN and HN lesions.

Performing such immunostaining was thus considered useful for enhancing the diagnostic accuracy of distinguishing lesions for which it is difficult to make a diagnosis due to the small amount of biological material or for lesions in which pathologists cannot reach an

agreement based on HE staining only.

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