Gut and Liver
https://doi.org/10.5009/gnl20195
pISSN 1976-2283 eISSN 2005-1212

Original Article

Histopathological Analysis of Esophageal Mucosa in Patients with Achalasia

Bong Eun Lee[1], Gwang Ha Kim[1], Nari Shin[2], Do Youn Park[3], and Geun Am Song[1]

[1]Department of Internal Medicine, Pusan National University School of Medicine and Biomedical Research Institute, Pusan National University Hospital, Busan, [2]Department of Pathology, Hanmaeum Changwon Hospital, Changwon, and [3]St. Maria Pathology Laboratory, Busan, Korea

Article Info
Received June 21, 2020
Revised September 29, 2020
Accepted October 10, 2020
Published online December 31, 2020

Corresponding Author
Gwang Ha Kim
ORCID https://orcid.org/0000-0001-9721-5734
E-mail doc0224@pusan.ac.kr

Background/Aims: Achalasia is an esophageal motor disorder that leads to functional esophageal obstruction. Food stasis and bacterial fermentation can predispose an individual to esophageal mucosal inflammation, causing multifocal dysplasia and increasing the risk of developing esophageal squamous cell carcinoma. We aimed to evaluate esophageal mucosal alterations in achalasia patients and determine clinical factors associated with the histopathological findings.

Methods: From 2009 to 2013, we obtained endoscopic biopsies from the lower and middle esophagus of 22 patients with achalasia and 17 controls. Patients' clinical data and histological severity of esophagitis were retrospectively analyzed. Additionally, immunohistochemical staining for CD3, CD20, Ki-67, and p53 was conducted.

Results: The median age of achalasia patients was 49.5 years (range, 27 to 82 years), and there were nine males (40.9%). The median symptom duration was 5.8 years (range, 1 to 33.5 years), and 10 patients (45%) underwent previous treatment (nine, balloon dilation; one, botulinum toxin injection). Achalasia patients had significantly more severe esophagitis than did controls (p=0.001, lower esophagus; p=0.008, middle esophagus), and the number of CD3-positive lymphocytes exceeded that of CD20-positive lymphocytes (p<0.001). Achalasia patients also had a higher esophageal Ki-67 proliferation index (p=0.048). Although statistically nonsignificant, p53 expression was only observed in achalasia patients. There was no association between the histological severity of esophagitis and other clinicopathological findings.

Conclusions: Achalasia patients showed significantly severe histological esophagitis and a high Ki-67 proliferation index, indicating an increased risk of neoplastic progression. Therefore, careful endoscopic inspection is necessary for the early detection of superficial neoplasia in these patients. **(Gut Liver 2021;15:713-722)**

Key Words: Esophageal achalasia; Esophageal neoplasms; Esophagitis; Proliferation marker Ki-67; Tumor suppressor gene p53

INTRODUCTION

Achalasia is a primary esophageal motility disorder that is characterized by the inability of the lower esophageal sphincter (LES) to relax in the absence of peristalsis, which is derived from degeneration or dysfunction of inhibitory neurons within the distal esophagus and LES.[1] Although the etiology of achalasia remains unclear, inflammation of the myenteric plexus is considered to be responsible for the functional loss of its ganglion cells.[1] Impaired transit of food through the esophagus can clinically manifest as dysphagia, heartburn, regurgitation, chest pain, weight loss, and respiratory symptoms. In the long term, chronic food stasis and bacterial fermentation can induce chronic hyperplastic esophagitis and dysplasia, which may eventually develop into esophageal squamous cell carcinoma (ESCC).[2] The prevalence of ESCC in achalasia patients reportedly ranges from 0.4% to 9.2%, with a considerable variation.[3-5]

Several researchers have evaluated esophageal mucosal alterations in achalasia.[6-12] Esophageal biopsy or esopha-

gectomy specimens were examined, and an overall marked squamous epithelial hyperplasia with some dysplastic changes was observed in most achalasia patients. Some studies evaluated the characteristics of inflammatory infiltrates based on immunohistochemical (IHC) staining for B or T lymphocytes, whereas some studies performed IHC staining for the proliferation marker Ki-67 and tumor suppressor genes such as p53, p21, and p16 to estimate the risk of ESCC in achalasia patients. However, these studies reported complicated results owing to different patient populations having variable disease status. Furthermore, the rarity of the disorder (which contributes to smaller study sample size) and different methods used for tissue acquisition and histologic interpretation can influence study outcomes. Therefore, in this study, we aimed to evaluate esophageal mucosal alterations in achalasia patients without concurrent neoplastic progression by comparing them with controls. We further aimed to evaluate the association between histopathologic results and clinical parameters in achalasia patients.

MATERIALS AND METHODS

1. Study population

From January 2009 to December 2013, we performed endoscopic esophageal biopsies in 22 achalasia patients without concurrent neoplasia and 17 patients who presented with dysphagia and/or reflux-like symptoms but were not diagnosed with specific esophageal disease including reflux esophagitis and eosinophilic esophagitis. Achalasia was diagnosed based on the results of endoscopy, conventional manometry, and barium esophagography. The control group was similarly evaluated, and all patients of the control group had no obvious abnormalities on endoscopy and esophagography. In their manometry, eight patients revealed ineffective esophageal motility (IEM) with 30% or greater of distal esophageal contraction amplitude of <30 mm Hg, whereas those of the other patients showed normal findings. Biopsy specimens were obtained from the lower and middle esophagus, separately fixed in 10% formalin, embedded in paraffin wax, and stained with hematoxylin and eosin stain for microscopic examination. Written informed consent was obtained from all participants before endoscopy, and this study was approved by the Institutional Review Board of the Pusan National University Hospital (IRB number: 1911-012-085).

2. Clinical data

We retrospectively reviewed patients' medical records to extract clinical data. In the achalasia group, symptom duration was calculated from symptomatic onset till the time of endoscopic examination, and the Eckardt symptom score[13] was used to assess the severity of symptoms. The presence and types of previous treatment for achalasia were also identified. One experienced endoscopist (B.E.L.) reviewed all endoscopic images to assess the grade of food stasis (grade 0=no retention; grade 1=saliva or liquid retention; grade 2=solid food retention), presence of esophageal candidiasis, and presence of esophageal mucosal thickening with white discoloration (Fig. 1). Maximal diameter of the esophageal body and presence of a sigmoid-shaped esophagus, and resting LES pressure were evaluated using barium esophagography and conventional manometry, respectively. A sigmoid-shaped esophagus was defined according to Japanese classification system for esophageal achalasia; based on barium esophagography, the bending of the esophagus at an angle of less than 135° was classified as a sigmoid-shaped esophagus.[14]

3. Histopathological analysis

The severity of esophagitis was assessed by two experienced gastrointestinal pathologists (N.S. and D.Y.P.) in a blind manner using the Ismail-Beigi histopathological criteria (Table 1).[15] These criteria included (1) basal cell hyperplasia; (2) elongation of papillae; (3) dilation of papil-

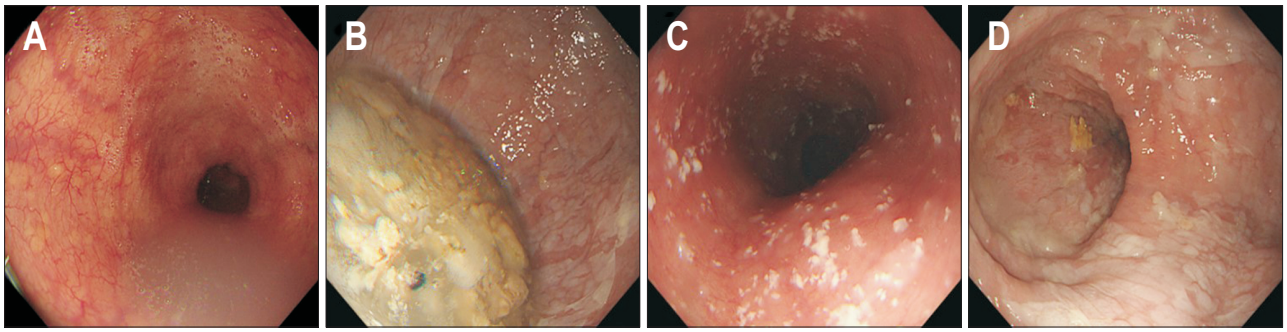

Fig. 1. Endoscopic findings of achalasia. (A) Saliva and liquid retention indicative of grade 1 food stasis is shown. (B) Image showing grade 2 food stasis constituting solid food retention. (C) Esophageal candidiasis. (D) Thickened esophageal mucosa with white discoloration.

lary vascular spaces; (4) intraepithelial inflammatory infiltration; (5) mucosal erosion; and (6) granulation tissue (Fig. 2). Fulfillment of criteria 1–3 indicated grade 1, and the presence of criterion 4 with or without 1 to 3 was defined as grade 2 esophagitis. Grade 3 esophagitis was confirmed with criteria 5 or 6. Although the Ismail-Beigi histopathological criteria was initially proposed for the evaluation of reflux esophagitis, none of these features is specific for reflux esophagitis and other causes of esophagitis also could reveal these histological changes. Lehman *et al*.[11] reported that the squamous mucosa in esophagectomy specimens of end-stage achalasia closely resembles that seen in gastroesophageal reflux disease, and we chose these criteria for the evaluation of esophageal chronic inflammation in achalasia patients.

Table 1. Histopathological Criteria and Grade of Esophagitis

Criteria
1. Basal cell hyperplasia
2. Elongation of papillae
3. Dilation of papillary vascular spaces
4. Intraepithelial inflammatory infiltration
5. Mucosal erosion
6. Granulation tissue
Grade
1. Presence of criteria 1–3
2. Presence of criterion 4 with or without 1–3
3. Presence of criteria 5 or 6

We performed IHC staining for detecting CD3, CD20, Ki-67 proliferation, and p53 expression in all specimens obtained from the lower and middle parts of the esophagus. However, two lower esophageal specimens from the control group and three middle esophageal specimens (two achalasia and one control group) were excluded from the analysis because of the unavailability of tissue samples. Selected 5-μm sections were deparaffinized and rehydrated. The sections were then submerged in citrate antigen retrieval buffer, microwaved for antigen retrieval, treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and then incubated with 1% bovine serum albumin to block nonspecific binding. Thereafter, the sections were incubated with CD3 antibody (SP7, 1:400; LabVision-NeoMarkers, Fremont, CA, USA), CD20 antibody (L26, 1:1000; Dako, Carpinteria, CA, USA), Ki-67 marker (MIB-1, 1:400; Dako), and p53 marker (SP5, 1:100; LabVision-NeoMarkers) at 4°C overnight. Normal rabbit serum was used as the negative control. Tissue sections were sequentially washed and treated with secondary antibody. The treated sections were counterstained with hematoxylin, dehydrated, and mounted. Normal lymph nodes with cytoplasm that tested positive for CD3 and CD20 and specimens of squamous cell carcinoma in which, nuclei tested positive for Ki-67 and p53, were used as positive controls. Adjacent normal squamous cells were used as the

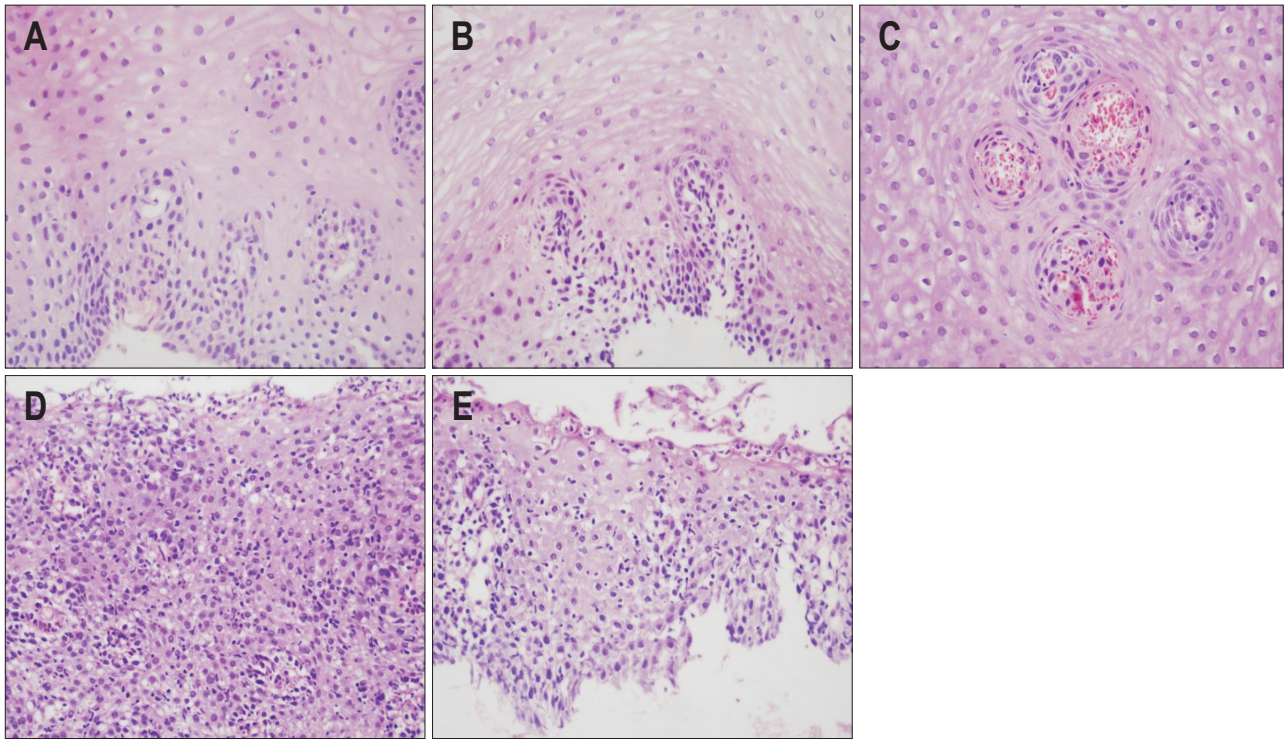

Fig. 2. Histopathological criteria of esophagitis. (A) Basal cell hyperplasia. (B) Elongation of papillae. (C) Dilation of papillary vascular spaces. (D) Intraepithelial inflammatory infiltration. (E) Mucosal erosion (H&E, ×100).

internal negative controls. The number of intraepithelial CD3- and CD20-positive lymphocytes per high-power field (HPF) were manually counted (Fig. 3). Ki-67 proliferation index (Fig. 4) and the degree of nuclear p53 expression (Fig. 5) were evaluated based on nuclear positivity of basal cells and they were graded as follows: 0=no staining; 1=rare basal cell staining; 2=extensive basal cell staining; and 3=suprabasilar staining.

4. Statistical analysis

Continuous variables are expressed as median (range), whereas categorical variables are presented as frequencies with percentages. The Mann-Whitney test was used to compare continuous variables, and the chi-square and Fisher exact tests were used for comparative analyses of categorical variables. Statistical analyses were performed using the SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) software. p-values <0.05 were considered to indicate statistical significance.

RESULTS

1. Baseline clinical characteristics

The median age of patients at the time of endoscopic examination was 49.5 years (range, 27 to 82 years) and 53 years (range, 22 to 71 years) in the achalasia group (n=22) and the control group (n=17), respectively (Table 2). Nine (40.9%) and six (47.1%) participants were men in the achalasia and control groups, respectively. In achalasia patients,

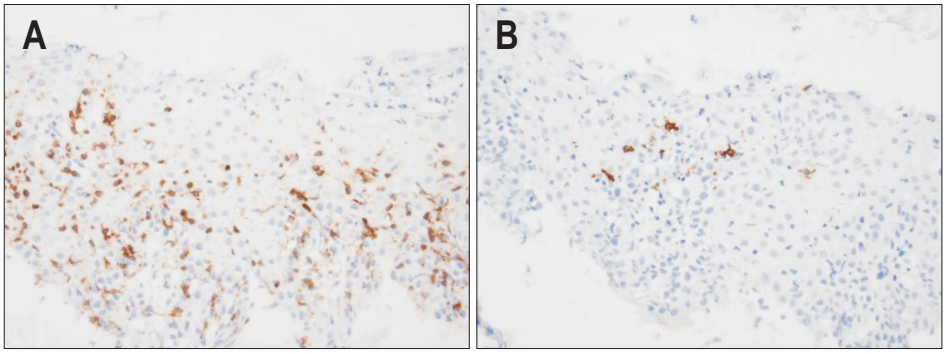

Fig. 3. Representative immunohistochemical staining for CD3 and CD20 in a patient with achalasia. (A) The CD3-positive lymphocyte count was 330/HPF. (B) CD20-positive lymphocytes were observed (number, 26/HPF) (CD3 and CD20 stain, ×100). HPF, high-power field.

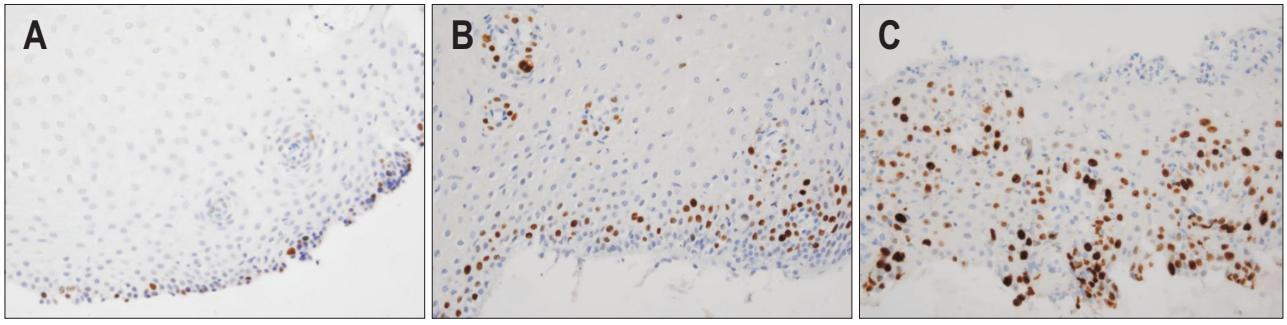

Fig. 4. Immunohistochemical staining for the Ki-67 proliferation index. (A) Grade 1: rare basal cell staining. (B) Grade 2: extensive basal cell staining. (C) Grade 3: suprabasilar staining (Ki-67 stain, ×100).

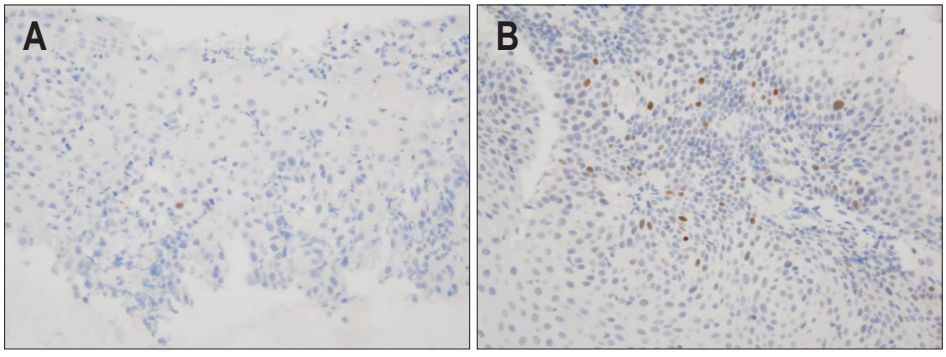

Fig. 5. Immunohistochemical staining for p53 expression. (A) Grade 0: no staining. (B) Grade 2: extensive basal cell staining (p53 stain, ×100).

Table 2. Baseline Clinical Characteristics

Characteristic	Achalasia group (n=22)	Control group (n=17)	p-value
Age, yr	49.5 (27–82)	53.0 (22–71)	0.812
Male sex	9 (40.9)	6 (47.1)	0.701
Symptom duration, yr	5.8 (1.0–33.5)	NA	
Eckardt score	5 (2–11)	NA	
Previous treatment	10 (45.5)	NA	
Balloon dilation	9 (90)		
Botox injection	1 (10)		
Maximal diameter of the esophageal body, mm	47.6 (22.2–58.0)	NA	
Sigmoid-shaped esophagus	11 (50.0)	NA	
LES resting pressure, mm Hg	21.3 (7.4–40.3)	NA	
Grade of food stasis		NA	
Grade 0	5 (22.7)		
Grade 1	15 (68.2)		
Grade 2	2 (9.1)		
Candida infection	3 (13.6)	NA	
Thickened esophageal mucosa with white discoloration	8 (36.4)	NA	

Data are presented as median (range) or number (%).
LES, lower esophageal sphincter; NA, not available.

the median symptom duration was 5.8 years (range, 1 to 33.5 years), and the median Eckardt score was 5 (range, 2 to 11). Overall, 10 patients underwent interventional treatment previously (nine, balloon dilation; one, botulinum toxin injection). The median maximal diameter of the esophageal body on barium esophagography was 47.6 mm (range, 22.2 to 58.0 mm), and the median LES resting pressure was 21.3 mm Hg (range, 7.4 to 40.3 mm Hg). Esophageal food stasis was observed in 17 patients (77.3%) (15 of grade 1 and two of grade 2). *Candida* infection and thickened esophageal mucosa with white discoloration were noted in three (13.6%) and eight (36.4%) patients, respectively. Meanwhile, during the 47.4 months of mean follow-up, there was no one diagnosed with esophageal dysplasia or cancer.

2. Histological grading of esophagitis in the achalasia and control groups

The severity of esophagitis based on the Ismail-Beigi histopathological criteria was significantly higher in the achalasia group than in the control group (Table 3). In the achalasia group, grades 1, 2, and 3 esophagitis of the lower esophagus was observed in four (18.2%), 16 (72.7%), and two (9.1%) patients, respectively. Comparatively, in the control group, only grades 1 and 2 esophagitis were observed in 13 (76.5%) and four (23.5%) patients, respectively (p=0.001). On examination for middle esophageal specimens, the achalasia group also demonstrated a higher grade of esophagitis (grades 1, 2, and 3 in 5 [22.7%] vs 13 [76.5%], 15 [68.2%] vs 4 [23.5%], and 2 [9.1%] vs 0 [0%] patients, respectively; p=0.002).

When subdividing the control group into two groups according to the manometric findings (IEM vs normal manometry), achalasia group also showed higher grade of esophagitis in both lower (p=0.001) and middle (p=0.037) esophagus than IEM group. And there was no significant difference in histological esophagitis between two subdivided control groups in both lower (p=0.576) and middle (p=1.000) esophagus.

Table 3. Histopathological Grade of Esophagitis in the Achalasia and Control Groups

Grade	Achalasia group (n=22)	Control group (n=17)	p-value
Lower esophagus, No. (%)			0.001
Grade 1	4 (18.2)	13 (76.5)	
Grade 2	16 (72.7)	4 (23.5)	
Grade 3	2 (9.1)	0	
Middle esophagus, No. (%)			0.002
Grade 1	5 (22.7)	13 (76.5)	
Grade 2	15 (68.2)	4 (23.5)	
Grade 3	2 (9.1)	0	

3. Inflammatory infiltrates, Ki-67 and p53 expression assessed immunohistochemistry in the achalasia and control groups

In the lower esophagus, the median number of intraepithelial CD3-positive lymphocytes was 93 (range, 0 to 570)/HPF and 63 (range, 39 to 147)/HPF in the achalasia and control groups, respectively (p=0.517), whereas the median number of CD20-positive lymphocytes was 0 (range, 0 to 145)/HPF and 1 (range, 0 to 4)/HPF in the achalasia and control groups, respectively (p=0.763) (Table 4). Although the number of CD3- and CD20-positive lymphocytes did

Table 4. Immunohistochemical Findings in the Lower and Middle Esophagus

Immunohistochemical findings	Lower esophagus			Middle esophagus		
	Achalasia group (n=22)	Control group (n=15)	p-value	Achalasia group (n=20)	Control group (n=16)	p-value
CD3-positive cells, /HPF	93 (0–570)	63 (39–147)	0.517	90 (0–420)	104 (0–325)	0.919
CD20-positive cells, /HPF	0 (0–145)	1 (0–4)	0.763	0 (0–26)	0 (0–56)	0.708
p53 Immunoreactivity			0.187			1.000
Grade 0	17 (77.3)	15 (100)		19 (95.0)	16 (100)	
Grade 1	4 (18.2)	0		1 (5.0)	0	
Grade 2	1 (4.5)	0		0	0	
Grade 3	0	0		0	0	
Ki-67 proliferation index						
Grade 0	3 (13.6)	2 (13.3)	0.048	5 (25.0)	7 (43.8)	0.471
Grade 1	7 (31.8)	11 (73.3)		7 (35.0)	7 (43.8)	
Grade 2	10 (45.5)	2 (13.3)		6 (30.0)	2 (12.5)	
Grade 3	2 (9.1)	0		2 (10.0)	0	
Grade 0–1	10 (45.5)	13 (86.7)	0.001	12 (60.0)	14 (87.5)	0.133
Grade 2–3	12 (54.5)	2 (13.3)		8 (40.0)	2 (12.5)	

Data are presented as median (range) or number (%).
HPF, high-power field.

not differ significantly between the two groups, the former significantly exceeded the latter among achalasia patients (p<0.001). The achalasia group showed significantly higher Ki-67 proliferation index than the control group (grades 0, 1, 2, and 3 in 3 [13.6%] vs 2 [13.3%], 7 [31.8%] vs 11 [73.3%], 10 [45.5%] vs 2 [13.3%], and 2 [9.1%] vs 0 [0%] patients, respectively; p=0.048). On dividing into two subgroups of grade 0–1 and grade 2–3, patients with grade 2–3 Ki-67 proliferation indices accounted for 54.5% of patients in the achalasia group, a proportion which was significantly greater than the 13.3% in the control group (p=0.001). When subdividing the control group into IEM and normal manometry groups, achalasia group also had significantly higher proportion of grade 2–3 Ki-67 proliferation index than two subdivided control groups (p=0.022). All patients in the control group and 17 (77.3%) in the achalasia group tested negative for p53 expression. Although statistically insignificant due to small number of cases, grade 1 and 2 expressions of p53 was observed in four (18.2%), and one (4.5%) patient, respectively, in the achalasia group (p=0.187), featuring preneoplastic process of achalasia.

In the middle esophagus, the median number of intraepithelial CD3- and CD20-positive lymphocytes was 90 (range, 0 to 420)/HPF and 0 (range, 0 to 26)/HPF in the achalasia group and 103.5 (range, 0 to 325)/HPF and 0 (range, 0 to 56)/HPF in the control group, respectively. These differences were not statistically significant (Table 4). The number of CD3-positive lymphocytes was higher than that of CD20-positive lymphocytes in the achalasia group (p<0.001). Both middle esophageal Ki-67 proliferation index (p=0.309) and p53 expression (p=1.000) did not differ significantly between the achalasia and control groups. All subjects, except one achalasia patient, showed negative p53 expression.

4. Clinical features and IHC findings in achalasia according to the histological severity of esophagitis in the lower and middle esophagus

In the analysis of the lower esophageal specimens, the median symptom duration did not differ between patients with grade 1 and those with grade 2–3 esophagitis (Table 5). Although the median maximal diameter of the esophageal body was larger in patients with grade 2–3 than in those with grade 1 esophagitis (49.5 [range, 27.0 to 58.0] mm vs 37.2 [range, 22.2 to 52.6] mm), this difference did not reach statistical significance (p=0.158). Endoscopic findings and IHC results did not correlate with the histological severity of esophagitis.

Furthermore, the histological severity of esophagitis in the middle esophagus also did not show any correlation with clinical features and IHC findings (Table 5).

DISCUSSION

In the present study, compared with controls, achalasia patients showed significantly more severe histological esophagitis of both the lower and middle esophagus and had higher Ki-67 proliferation in the lower esophagus. The number of CD3-positive lymphocytes significantly exceeded that of CD20-positive lymphocytes in achalasia patients, although the CD3-positive lymphocyte count did not dif-

Table 5. Clinical Features and Immunohistochemical Findings in Patients with Achalasia According to the Histological Severity of Esophagitis in the Lower and Middle Esophagus

Variable	Lower esophagus			Middle esophagus		
	Grade 1 (n=4)	Grade 2–3 (n=18)	p-value	Grade 1 (n=5)	Grade 2–3 (n=17)	p-value
Age, yr	51.5 (44–47)	47.5 (27–82)	0.774	44.0 (43–57)	53.0 (27–82)	0.543
Male sex	1 (25.0)	8 (44.4)	0.616	2 (40.0)	7 (41.2)	1.000
Symptom duration, yr	5.8 (3–11)	6.8 (1–33.5)	0.859	11.0 (3–12)	5.1 (1–33.5)	0.768
Previous treatment	2 (50.0)	8 (44.4)	1.000	3 (60.0)	7 (41.2)	0.624
Maximal diameter of the esophageal body, mm	37.2 (22.2–52.6)	49.5 (27.0–58.0)	0.158	42.9 (22.2–52.6)	47.6 (27.0–58.0)	0.382
Sigmoid-shaped esophagus	1 (25.0)	10 (55.6)	0.586	2 (40.0)	9 (52.9)	1.000
Grade of food stasis			1.000			1.000
Grade 0	1 (25.0)	4 (22.2)		1 (20.0)	4 (23.5)	
Grade 1	3 (75.0)	12 (66.7)		4 (80.0)	11 (64.7)	
Grade 2	0	2 (11.1)		0	2 (11.8)	
Candida infection	0	3 (16.7)	1.000	0	3 (17.6)	1.000
Thickened esophageal mucosa with white discoloration	2 (50.0)	6 (33.3)	0.602	3 (60.0)	5 (29.4)	0.309
p53 Immunoreactivity			0.628			1.000
Grade 0	4 (100)	13 (72.2)		4 (100)*	15 (93.8)*	
Grade 1	0	4 (22.2)		0	1 (6.0)	
Grade 2	0	1 (5.6)		0	0	
Grade 3	0	0		0	0	
Ki-67 proliferation index			0.627			0.471
Grade 0	1 (25.0)	2 (11.1)		2 (50.0)*	3 (18.8)*	
Grade 1	2 (50.0)	5 (27.8)		2 (50.0)	5 (31.3)	
Grade 2	1 (25.0)	9 (50.0)		0	6 (37.5)	
Grade 3	0	2 (11.1)		0	2 (12.5)	

Data are presented as median (range) or number (%).
*Two middle esophageal specimens in patients with achalasia were excluded from the analysis because of the unavailability of tissue samples.

fer significantly between the achalasia and control groups. Although statistically insignificant, the p53 expression was only observed in the achalasia patients. Meanwhile, no association observed between the histological severity of esophagitis and other clinical and IHC features. This study is worthwhile as the first research identifying esophageal mucosal inflammation including both p53/Ki-67 expression and characterization of lymphocytic infiltration using biopsy specimens in achalasia patients, and we separately analyzed the mucosal alteration according to each level of esophagus (lower and middle).

Achalasia is considered a premalignant condition for ESCC, and chronic hyperplastic esophagitis due to food retention and bacterial overgrowth is expected to incite malignant transformation of esophageal squamous epithelium via an inflammation-dysplasia-carcinoma sequence.[2,3] Although the reported risk of ESCC in achalasia varies widely according to different studies, ranging from 0.4% to 9.2%,[3] several autopsy-based studies have reported the prevalence of esophageal neoplasia in affected patients to be >20%.[8]

Some trials have documented the mucosal morphological alterations along with the abnormal status of cell proliferation and tumor suppressor genes, thus providing supportive evidence for such a carcinogenic pathway in achalasia.[6-11] In a study by Goldblum *et al.*,[6] surgically resected esophageal specimens from achalasia patients showed diffuse squamous hyperplasia and lymphocytic inflammation of the lamina propria and submucosa. Chino *et al.*[7] also reported marked hyperplastic changes in the stratified squamous epithelium along with multiple foci of dysplastic changes in resected esophageal specimens of achalasia patients and suggested that ESCC in achalasia patients was closely associated with dysplastic changes. Leeuwenburgh *et al.*[8] documented the overexpression of the tumor suppressor gene p53, as a predictor for the development of esophageal cancer in achalasia. In their research, the Ki-67 expression level was highly positive in achalasia patients, regardless of the concomitant presence of neoplasia (dysplasia or cancer), while the proportion of p53 overexpression was significantly higher in achalasia patients with concurrent dysplasia/cancer than in achalasia patients without neoplasia. Fujii *et al.*[9] also suggested that pathological proliferative states based on stronger nuclear expression of Ki-67 are possible stepping stones for esophageal carcinogenesis in achalasia patients. The

overexpression of the p53 protein was not observed in the absence of neoplasia. We also identified high Ki-67 proliferation within the lower esophageal tissue, and no p53 overexpression was observed in achalasia patients without concurrent neoplastic changes, which corroborated the findings of previous studies.[8,9] We analyzed two separate samples each from the lower and middle esophagus in each patient, and hyperproliferation was found only in the lower esophagus but not in the middle portion in achalasia patients, which was to be expected considering that the lower esophagus is more prone to irritation owing to food stasis and fermentation and thus is more susceptible to neoplastic transformation. This might be clinically supported by a recent meta-analysis, which reported that the majority of cases of achalasia-associated ESCC presented in the lower third of the esophagus (42%), followed by the middle third of the esophagus (40%), and the upper third of the esophagus (17%).[16] Meanwhile, we did not find any correlation between clinical factors and histopathological abnormalities. One recent study reported somewhat different results. Kim *et al.*[10] found that the rate of p53 expression was significantly higher in achalasia patients with retention esophagitis than in achalasia patients without retention esophagitis or in controls, even in the absence of concurrent neoplasia. Conversely, no significant difference in the expression of Ki-67 was observed between achalasia patients and controls.[10] Considering overall reported data, squamous mucosal alterations and high Ki-67 expression have been generally noted in achalasia patients, irrespective of its concurrent association with neoplastic changes. The overexpression of p53 has demonstrated increased association with neoplasia itself, although it is an uncommon feature in patients without neoplastic pathology.

In addition, several studies have descriptively characterized esophageal mucosal inflammation in achalasia.[11,12] Lymphocytic esophagitis was anecdotally noted in a study by Goldblum *et al.*[6] and is considered a morphologic change occurring secondary to food stasis-induced chronic inflammation. Lehman *et al.*[11] showed a higher number of CD3-positive cells in esophagectomy specimens from end-stage achalasia patients, and Döhla *et al.*[12] reported a T-cell-rich inflammatory response predominantly composed of CD4-positive cells with only few CD20-positive B cells in achalasia patients; this observation was corroborated by our results. However, subsequent studies concluded that this feature could not be considered pathognomonic of achalasia because esophageal epithelial inflammation in this disorder seems to be reactive and unspecific in nature.[12] Furthermore, there was a recent study that showed a significant increase of intraepithelial T lymphocytes in nonerosive reflux disease compared to normal controls.[17] This might be related with the failure to prove the significant difference of CD3-positive lymphocyte count between the achalasia and control groups, since we included controls with esophageal symptoms who had some possibilities for nonerosive reflux disease.

Esophageal cancers are rarely detected at an early stage in achalasia patients.[8,9] Achalasia patients are used to symptoms associated with impaired food passage and therefore tolerate them in the early stages, which leads to delayed diagnosis of esophageal cancer. Furthermore, endoscopic surveillance for ESCC is often difficult in achalasia patients because stasis or mucosal adherence of food and thickened irregular mucosal surface in a sigmoid-shaped esophagus (in long-standing) interferes with an early detection of neoplasia. It is possible that multifocal neoplastic foci with unclear borders may be present. Esophageal squamous hyperplasia and increased Ki-67 expression, considered as initial steps in carcinogenesis in achalasia patients, have been documented in most studies and in our research. Although, the vast majority of achalasia patients do not develop carcinoma, and currently, there is no consensus on using biomarkers and imaging surveillance for predicting ESCC in achalasia patients, thorough endoscopic inspection is necessary for the early detection of superficial neoplasia in achalasia patients. And the possibility of combined neoplasia should be especially considered in patients with an overexpression of p53.

Our study has some limitations. First, this study was based on a retrospective review of data. However, the study protocol had been designed before the collection of esophageal biopsy specimens from unselected patients (we consecutively enrolled achalasia patients during the study period), and we attempted to gather required information for the complete cohort. Second, we could not enroll healthy volunteers as controls and some controls showed IEM features on their esophageal manometry. However, they did not reveal any abnormalities on endoscopy and esophagography including mucosal breaks, and resultantly, even controls with IEM showed similar histological features to controls without IEM. Therefore, we could consider these patients as near normal. Third, we included achalasia patients with previous interventional treatment. In patients with successful treatment, reflux rather than retention could be the cause for histological esophagitis. However, whatever the cause of esophagitis was, we realized that achalasia patients had significant mucosal hyperproliferation. Absence of high-resolution manometry and unavailability of timed barium esophagography might be another limitation. Last, the rare occurrence of the disease itself limits our study owing to which we still cannot provide precise clinical recommendations based on

our findings. Nevertheless, we found that our results were consistent with those of previous studies identifying both tumor-related biomarkers and characterization of lymphocytic infiltration at each level of esophagus, and therefore, we can suggest and support our conclusions with stronger evidence.

In conclusion, we determined that achalasia was associated with significantly more severe histological esophagitis and had higher Ki-67 proliferation in the lower esophagus than controls. These features represent significant esophageal mucosal hyperproliferation, which indicates an increased risk of developing esophageal carcinoma in affected patients. Careful and thorough endoscopic surveillance might be necessary for the early detection of superficial neoplasia in achalasia patients. A further large-scale prospective study is warranted to determine any reliable biomarkers for estimating the risk of esophageal cancer and benefits and applicability of endoscopic surveillance in achalasia patients.

CONFLICTS OF INTEREST

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

ACKNOWLEDGEMENTS

This study was supported by the Medical Research Center Program through the National Research Foundation Grant funded by the Korean Government (NRF-2015R1A5A2009656).

AUTHOR CONTRIBUTIONS

Conceptualization: B.E.L., G.H.K. Methodology: B.E.L., G.H.K., N.S., D.Y.P. Formal analysis: B.E.L. Writing - original draft: B.E.L. Writing - review and editing: B.E.L., G.H.K., D.Y.P., G.A.S. Approval of final manuscript: all authors.

ORCID

Bong Eun Lee https://orcid.org/0000-0003-2734-2134
Gwang Ha Kim https://orcid.org/0000-0001-9721-5734
Nari Shin https://orcid.org/0000-0001-8743-3615
Do Youn Park https://orcid.org/0000-0001-7641-1509
Geun Am Song https://orcid.org/0000-0003-2100-8522

REFERENCES

1. Pandolfino JE, Gawron AJ. Achalasia: a systematic review. JAMA 2015;313:1841-1852.
2. Leeuwenburgh I, Haringsma J, van Dekken H, Scholten P, Siersema PD, Kuipers EJ. Long-term risk of oesophagitis, Barrett's oesophagus and oesophageal cancer in achalasia patients. Scand J Gastroenterol Suppl 2006;(243):7-10.
3. Torres-Aguilera M, Remes Troche JM. Achalasia and esophageal cancer: risks and links. Clin Exp Gastroenterol 2018;11:309-316.
4. Leeuwenburgh I, Scholten P, Alderliesten J, et al. Long-term esophageal cancer risk in patients with primary achalasia: a prospective study. Am J Gastroenterol 2010;105:2144-2149.
5. Gillies CL, Farrukh A, Abrams KR, Mayberry JF. Risk of esophageal cancer in achalasia cardia: a meta-analysis. JGH Open 2019;3:196-200.
6. Goldblum JR, Whyte RI, Orringer MB, Appelman HD. Achalasia: a morphologic study of 42 resected specimens. Am J Surg Pathol 1994;18:327-337.
7. Chino O, Kijima H, Shimada H, et al. Clinicopathological studies of esophageal carcinoma in achalasia: analyses of carcinogenesis using histological and immunohistochemical procedures. Anticancer Res 2000;20(5C):3717-3722.
8. Leeuwenburgh I, Gerrits MM, Capello A, et al. Expression of p53 as predictor for the development of esophageal cancer in achalasia patients. Dis Esophagus 2010;23:506-511.
9. Fujii T, Yamana H, Sueyoshi S, et al. Histopathological analysis of non-malignant and malignant epithelium in achalasia of the esophagus. Dis Esophagus 2000;13:110-116.
10. Kim H, Park H, Choi H, et al. Retention esophagitis as a significant clinical predictor of progression to esophageal cancer in achalasia. Clin Endosc 2018;51:161-166.
11. Lehman MB, Clark SB, Ormsby AH, Rice TW, Richter JE, Goldblum JR. Squamous mucosal alterations in esophagectomy specimens from patients with end-stage achalasia. Am J Surg Pathol 2001;25:1413-1418.
12. Döhla M, Leichauer K, Gockel I, et al. Characterization of esophageal inflammation in patients with achalasia: a retrospective immunohistochemical study. Hum Pathol 2019;85:228-234.
13. Eckardt VF, Aignherr C, Bernhard G. Predictors of outcome in patients with achalasia treated by pneumatic dilation. Gastroenterology 1992;103:1732-1738.
14. Japan Esophageal Society. Descriptive rules for achalasia of the esophagus, June 2012: 4th edition. Esophagus 2017;14:275-289.
15. Ismail-Beigi F, Horton PF, Pope CE 2nd. Histological consequences of gastroesophageal reflux in man. Gastroenterology 1970;58:163-174.
16. Tustumi F, Bernardo WM, da Rocha JRM, et al. Esophageal

achalasia: a risk factor for carcinoma: a systematic review and meta-analysis. Dis Esophagus 2017;30:1-8.
17. Mastracci L, Bruzzone M, Pacella E, et al. The contribution of intraepithelial inflammatory cells to the histological diagnosis of microscopic esophagitis. Esophagus 2016;13:80-87.