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Original article

Histopathological analysis of non-malignant and malignant epithelium in achalasia of the esophagus

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SUMMARY. **We studied the premalignant nature of achalasia using anti-Ki-67 and anti-p53 monoclonal antibodies immunohistochemically. In this study, four patients with esophageal carcinoma and achalasia were investigated. Three tumors were pT4 (UICC pTNM) and one tumor was pT1. The majority of non-malignant esophageal epithelium showed esophagitis and/or dysplasia histologically. Esophageal epithelial cells in the lesions of esophagitis and/or dysplasia had a higher number of Ki-67-positive cells than normal epithelial cells. p53 protein was expressed in two tumors and it was not expressed in non-malignant epithelium. From these results, we found that esophageal epithelium in achalasia lesions is changed to varying degrees of esophagitis and/or dysplasia by stagnation of intake foods, and these abnormal epithelial cells showed a high proliferative state compared with the normal cells without the *p53* gene mutation. We suggest that the distinct proliferative status is a cause of carcinogenesis.**

INTRODUCTION

Since the first description by Fagge[1] in 1872 of carcinoma developing in a patient with esophageal achalasia, this has been considered a major risk factor for esophageal carcinoma.[2] The reported incidence of concomitant carcinoma and achalasia ranges from 0.53% to 8.6%.[3–6] Although many investigators have warned clinicians about the high incidence of carcinogenesis in achalasia,[7] the prognosis of patients who develop these carcinomas remains poor. In patients with achalasia, carcinomas are rarely detected at an early stage because symptoms of esophageal carcinoma such as dysphagia, vomiting and pain on swallowing are often attributed to this disease.[8] When most patients with achalasia present with worsening of symptoms and an esophageal carcinoma, the tumor is often in an advanced stage and is frequently unresectable.[9] The explanation for the carcinogenesis in achalasia patients is unclear. Rake[10] postulated that the malignant transformation resulted from chronic irritation of the esophageal mucosa by retained food particles and saliva. The ensuing acute esophagitis produced by the stagnant material leads to ulceration and attempts to repair the damage. Areas of irregular epithelial hyperplasia observed in these damaged areas are thought to be a nidus for malignant transformation. To date, biomarkers associated with a risk of carcinogenesis have not been evaluated in patients with esophageal achalasia.

In this paper, we studied four achalasia patients with concomitant esophageal carcinoma. To assess the cause of carcinogenesis in esophageal achalasia, a clinicopathologic study was performed which included evaluation of proliferative status in non-malignant epithelium and cancer lesions by immunohistochemical analysis using monoclonal antibodies against Ki-67 nuclear antigen and p53 protein.

MATERIALS AND METHODS

Between 1982 and 1994, four achalasia patients with primary esophageal carcinoma underwent radical surgery. Preoperative 30-Gy radiation therapy was carried out for one patient (case 1) and the other three patients received no preoperative treatment. One of the four tumors was a superficial carcinoma (pT1N0M0), whereas the other three were in advanced stage (pT4N0M0 or pT4N1M0) according to the UICC TNM classification.[11] Surgically removed esophagus was fixed in 10% formalin for

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about 4 days and the whole fixed material was sectioned into 4×0.5 cm blocks. The blocks were paraffin-embedded and sliced to 3 μm thick for histologic and immunohistochemical studies. For histologic examination, all sections were stained with hematoxylin and eosin (HE) and non-malignant epithelium was divided into esophagitis, dysplasia and normal mucosa. Inflammatory epithelium (esophagitis) was identified by the presence of numerous inflammatory cells in the mucosal layer. Dysplasia was also characterized by the presence of abnormal cells which involved the basal layer and extended into the remaining normal epithelial cells. The areas of abnormal epithelium and cancer lesion were schematically presented in each case. Clinicopathologic features were analyzed according to patient age, gender, achalasia grade, progression to cancer (durations between achalasia and carcinoma), tumor location, pTNM, degree of cancer differentiation, lymphatic invasion, vascular invasion, intramural skip metastasis, synchronous multiple cancer and survival after surgery.

The staging of achalasia was determined according to the following Descriptive Rules for Achalasia described by the Japanese Society for Esophageal Diseases.[12] Grade I, diameter <3.5 cm; grade II, diameter 3.5 cm to <6 cm; and grade III, diameter ≥ 6 cm. The maximum diameter of the thoracic esophagus was measured on a standard esophagogram.

For evaluation of decreases in or absence of ganglion cells in the myenteric plexus, the number of ganglion cells was determined in 10 randomly selected sections of each case and was expressed as mean $\pm$ standard deviation (SD) per section.

All results obtained were compared with those of a control group consisting of 10 removed primary esophageal cancers without achalasia. The thickness of the muscularis propria, expressed as the mean $\pm$ SD per section, was also determined for each study case and the results were compared with those of the control group.

Cell proliferation in cancer lesions and non-malignant esophageal epithelium (normal, inflammatory and dysplastic) was evaluated by immunohistochemistry using two monoclonal antibodies (mAb) MIB-1 (Immunotech, Marseilles, France) for Ki-67 nuclear antigen and DO-7 (Dako, Glostrup, Denmark) for p53 protein. mAb MIB-1 for Ki-67 nuclear antigen is associated with cell proliferation, is expressed throughout the G_1 to M cell cycle phases and is absent in resting G_0 cells.[13] mAb DO-7 detects wild-type and mutant forms of p53 protein.[14] The *p53* gene encodes a 53-kDa nuclear phosphoprotein which controls the transition of cells from G_0/G_1 to the S-phase.[15] All sections for MIB-1 and DO-7 studies were heated in 10 mM citrate buffer for 15 min using a microwave oven before staining.

A LSAB Kit (Dako, Carpinteria, CA, USA) which utilizes the labeled streptavidin–biotin (LSAB) method was used for all immunohistochemical analyses. Before these mAb reactions, endogenous peroxidase activity was blocked by incubating sections in methanol with 3% hydrogen peroxide for 5 min and non-specific staining was blocked by incubating sections with carrier protein for 5 min. Specimens were then incubated with mAb MIB-1 for 2 h at room temperature and mAb DO-7 overnight at 4°C, followed by sequential 10-min incubations with biotinylated anti-mouse IgG and peroxidase-labeled streptavidin. Staining was completed after a 10-min incubation with substrate chromogen solution. Cells with staining of their nuclear region were considered to be Ki-67 or p53 positive. The Ki-67-positive cell rate (Ki-67 PR) was calculated for each specimen by counting the number of reactive cells per 1000 malignant or non-malignant esophageal epithelial cells in five randomly selected areas using a cell counter (OL-501A Weltech, Japan). A p53-positive tumor was defined as when p53 reactive cells were more than 20% of all tumor cells. The Ki-67 PR and p53-positive tumors were determined by two experienced observers who were blinded to each result. Ki-67 and p53 immunoreactivities were also examined in normal epithelium, inflammatory epithelium (esophagitis), dysplasia and cancer lesions.

Statistical differences were analyzed using the chi-squared test and Fisher's exact probability test as well as the Student *t*-test. Significant differences were considered when $p < 0.05$.

RESULTS

Clinical features

A clinical summary of the four patients with esophageal achalasia who developed squamous cell carcinoma is shown in Table 1. The two male and two female patients ranged in age from 43 to 63 years old (mean 53.5 $\pm$ 7.2). The grade of the achalasia was II in two patients and III in two patients. The interval between the first diagnosis of achalasia and carcinoma development ranged from 16 to 30 years (median 22.3 $\pm$ 6.1 years). The esophageal tumors were primarily located in the upper thoracic esophagus in three cases and in the middle thoracic esophagus in one case. Three of these patients died of cancer recurrences, one 7, one 2.5 and one 5 months after surgery. The one patient with superficial carcinoma who at the time of writing this report is alive 80 months after surgery shows no evidence of relapse.

Histopathologic results

A pathologic summary of these four cases is provided in Table 2. One tumor (case 3) was pT1N0M0, two

Table 1. Clinical summary of the four study patients

Cases	Age	Gender	Grade of achalasia*	Progression to cancer (years)†	Tumor location	Survival (months)‡	Prognosis
1	63	F	II	16	Upper	7	Dead
2	52	F	II	20	Upper	3	Dead
3	43	M	III	23	Middle	80	Alive
4	56	M	III	30	Upper	5	Dead

*Grade of achalasia was determined according to the Descriptive Rules for Achalasia of the Esophagus from the Japanese Society of Esophageal Diseases based on the maximal diameter of the thoracic esophagus on a standard esophagogram: grade I, diameter < 3.5 cm; grade II, diameter 3.5 to < 6 cm; grade III, diamter ≥6 cm.
†The interval between the diagnosis of achalasia and cancer development.
‡Months after surgery.

Table 2. Pathologic summary of the four study patients

Cases	pTNM*	Differentiation of the tumor†	Lymphatic invasion	Vascular invasion	Intramural metastasis	Multiple cancer
1	pT4N0M0	Moderately differentiated SCC	(+)	(−)	(−)	(−)
2	pT4N0M0	Moderately differentiated SCC	(+)	(+)	(−)	(−)
3	pT1N0M0	Well differentiated SCC	(−)	(−)	(−)	(+)
4	pT4N1M0	Well differentiated SCC	(+)	(+)	(−)	(−)

*The interval between the diagnosis of achalasia and cancer development. According to the UICC pTNM classification.
†All tumors were squamous cell carcinomas (SCC).

tumors were pT4N0M0 (cases 1 and 2) and one tumor was pT4N1M0 (case 4). All four tumors were squamous cell carcinomas (SCC) involving two moderately differentiated and two well-differentiated types. All pT4 tumors showed a significant vessel invasion but no intramural skip metastasis was detected. Case 3 had a synchronous multiple esophageal cancer.

There was a significant difference ($p < 0.01$) in the mean number of myenteric plexus ganglion cells between achalasia patients with esophageal carcinoma (7.6 ± 2.7) and control patients (21.7 ± 6.1). There was also a significant difference ($p < 0.01$) in the thickness of the muscularis propria between the two patient groups, with the values of 5.2 ± 1.6 mm and 2.4 ± 0.6 mm respectively (Table 3).

Figures 1–4 show a schematic presentation reconstructed by the pathological extent of carcinoma, esophagitis, dysplasia lesions and normal epithelium. Case 1, whose achalasia grade was I, showed several small lesions of esophagitis, dysplasia and carcinoma coexisting in normal esophageal epithelium. In cases 2 and 3, most of the esophageal epithelium was occupied by esophagitis or dysplasia. In case 4 the majority of the epithelium consisted of esophagitis and dysplasia except for a massive carcinoma. The esophageal epithelium was changed to abnormal lesions in all achalasia patients.

Table 3. Number of ganglion cells in the myenteric plexus and thickness of the muscularis propria

Cases	Number of ganglion cells (counts)†	Thickness of muscularis propria (mm)‡
Achalasia with esophageal cancer group	7.6 ± 2.7 *	5.2 ± 1.6 *
Control group (esophageal cancer alone)	21.7 ± 6.1 *	2.4 ± 0.6 *

*$p < 0.01$.
Results are compared with those of a control group who had primary esophageal carcinoma without achalasia.
†The number of ganglion cells in the myenteric plexus was determined in 10 sections for each case.
‡The thickness of a muscularis propria was also measured in 10 sections for each case.

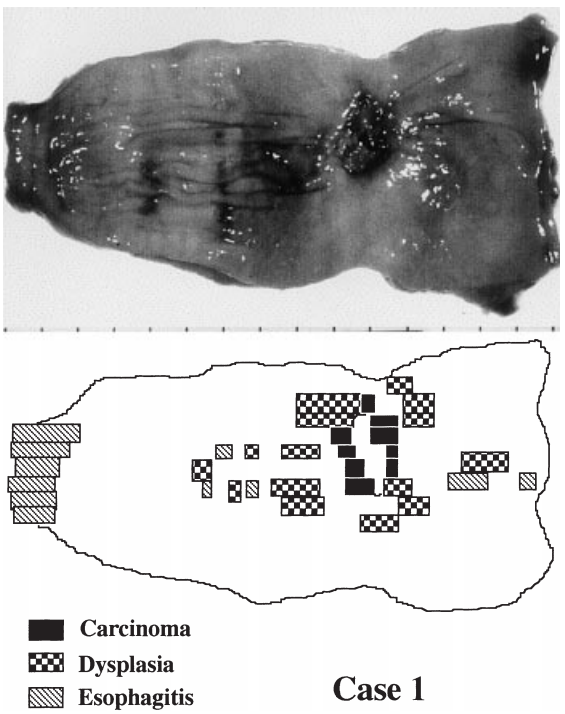

Fig. 1—Histopathological appearance in case 1. Localized esophagitis and dysplasia were developed in the non-malignant esophageal epithelium.

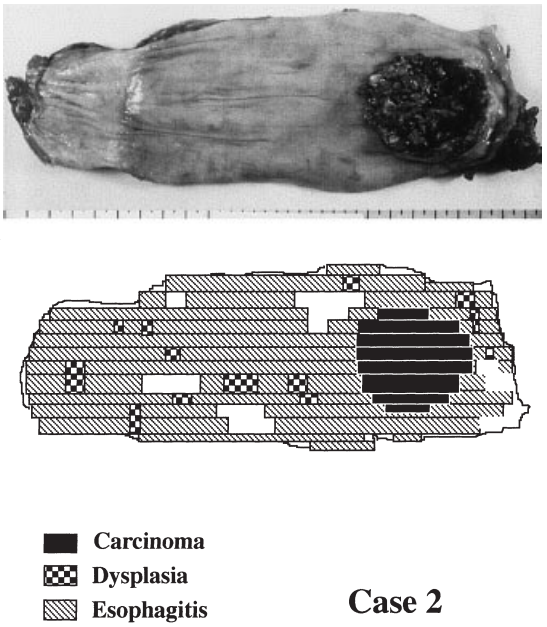

Fig. 2—Histopathological appearance in case 2. The non-malignant esophageal epithelium was occupied by esophagitis.

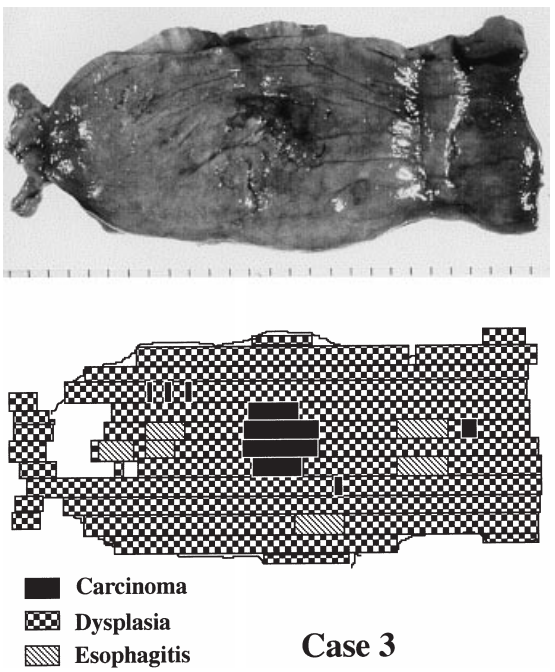

Fig. 3—Histopathological appearance in case 3. The majority of the non-malignant epithelium was changed to dysplasia.

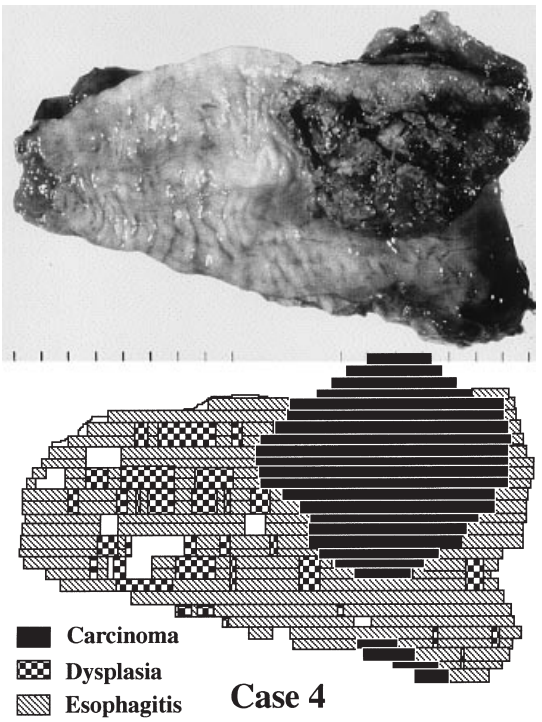

Fig. 4—Histopathological appearance in case 4. Most non-malignant epithelium was changed to dysplasia and esophagitis.

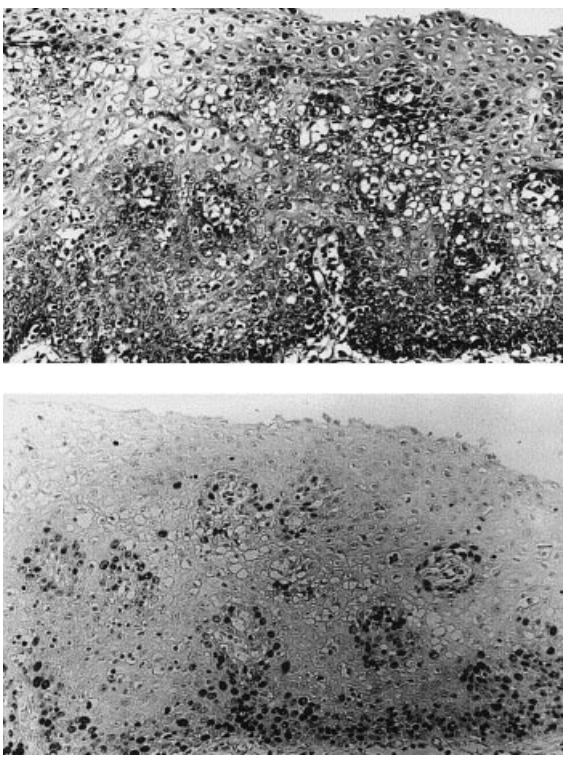

Fig. 5—Histological findings in inflammatory epithelium that was routinely stained with HE and mAb MIB-1 immunohistochemistry (case 4). Larger numbers of lymphocytes are present in the esophageal epithelium. Ki-67-positive cells were found in the proliferating cells.

Immunohistochemistry

Figure 5 shows a lesion of esophagitis (HE staining) and its Ki-67-positive cells. Figure 6 also shows a dysplasia lesion.

The Ki-67-positive cell rate in normal epithelium, esophagitis, dysplasia and cancer lesions is shown in Table 4.

The average Ki-67 PR showed a significant high value ($p < 0.01$) in esophagitis (13.6 ± 3.6) or dysplasia (21.8 ± 3.6) compared with normal

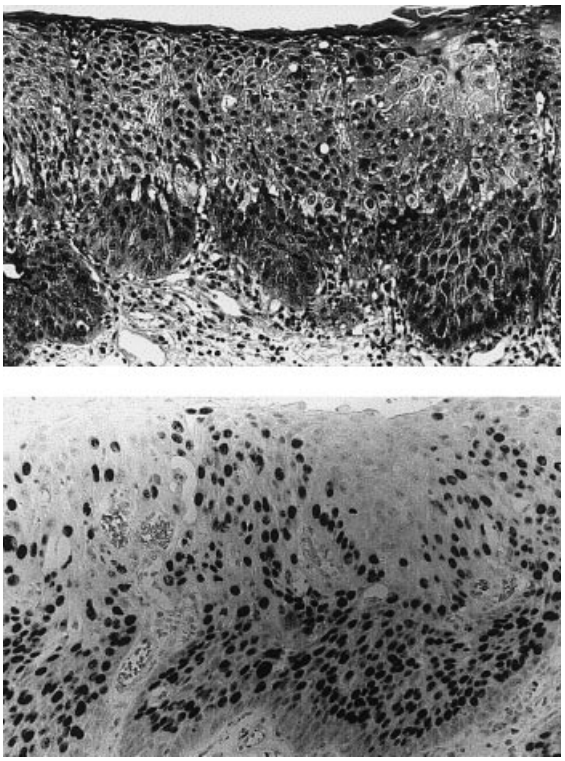

Fig. 6—Histological findings in dysplasia that was stained with HE and mAb MIB-1 immunohistochemistry (case 3). High-grade dysplasia was found showing cellular crowding and nuclear pleomorphism affected two-thirds of the epithelium. Numerous cells in the dysplasia lesion strongly expressed Ki-67 antigen.

epithelium (4.6 ± 1.8). Furthermore, cancer cells had the highest Ki-67 reactivity (32.3 ± 5.7). Positive expression of p53 protein was found in two tumors (cases 2 and 3) but that was not recognized in normal esophageal epithelium, esophagitis or dysplasia lesions (Table 5).

DISCUSSION

Achalasia is a motor disorder of the esophagus characterized by failure of relaxation of the lower esophageal sphincter and a lack of progressive peristalsis in the esophageal body.[16] Pathologic descriptions have demonstrated a loss of ganglion cells in the myenteric plexus in primary achalasia.[17, 18] Goldblum et al[19] studied 42 patients who underwent esophagectomy for achalasia and, in all cases, myenteric ganglion cells within the esophageal body were markedly diminished and muscular hypertrophy of the muscularis propria was seen. We also found a significant difference in the mean number of ganglion cells in the myenteric plexus of the esophagus between achalasia patients with esophageal cancer and control patients, as well as a significant difference in the thickness of the muscularis propria between the two patient groups. Based on the histologic result, our four patients were diagnosed with achalasia.

Achalasia, Barrett's esophagus[20] and lye strictures[21] have been considered an important risk factor for esophageal carcinoma.[2] The incidence of carcinoma in patients with achalasia has been reported to be 8.6% by Lortat-Jacob et al,[5] 0.53% by Wychulis et al,[6] 6.6% by Camara-Lopes[3] and 3% by Just-Viera and Haight.[4] In a recent study, Meijssen et al[22] reported that the risk of developing squamous cell carcinoma in achalasia patients was increased 33-fold over that in the general population. Just-Viera and Haight[4] suggested that food retention, increased bacterial growth and chemical irritation present in achalasia and chronic esophagitis may render the epithelium more sensitive to carcinogens. Goldblum et al[19] noted several epithelial abnormalities in achalasia, one patient had a focus of high-grade squamous dysplasia, one had superficial squamous cell carcinoma and 28 had lymphocytic esophagitis. Further, diffuse squamous hyperplasia was seen in all cases. In our four patients, we found similar abnormalities in the esophageal epithelium. Most of the epithelium was replaced by dysplasia or esophagitis in three patients, whereas small areas of esophagitis and dysplasia were found to coexist in one patient.

To investigate the degree of cell proliferation, malignant and non-malignant epithelium was stained with mAb MIB-1. MIB-1 reacts with the Ki-67 nuclear antigen. Ki-67 nuclear antigen is weakly expressed in the S-phase, but it progressively increases through the S and G_2 phases to reach a maximum level at mitosis. After division, the cells return to the G_1 phase in which Ki-67 levels progressively decrease and then finally disappear when cells enter the long

Table 4. Ki-67-positive cell rate in four study patients

Cases	Normal epithelium	Esophagitis	Dysplasia	Cancer
1	3.0 ± 1.3	8.9 ± 1.6	17.5 ± 1.6	24.5 ± 1.3
2	4.5 ± 1.6	14.8 ± 2.9	22.1 ± 2.3	36.3 ± 4.1
3	6.5 ± 0.7	14.1 ± 2.4	24.8 ± 2.4	35.2 ± 3.7
4	4.2 ± 1.3	16.7 ± 1.7	22.7 ± 3.1	33.0 ± 3.8
Mean ± SD	4.6 ± 1.8	13.6 ± 3.6	21.8 ± 3.6	32.3 ± 5.7

* $p < 0.01$.
Positive staining of the nuclear region inside tumor cells or non-malignant cells was considered Ki-67 positive. Ki-67-positive cell rate (PR) was calculated by measuring the number of positive cells per 1000 cells in five areas for each specimen.

Table 5. p53 protein expression in four study patients

Cases	Normal epithelium	Esophagitis	Dysplasia	Cancer
1	–	–	–	–
2	–	–	–	+
3	–	–	–	+
4	–	–	–	–

Tumors were considered positive when more than 20% of all tumor cells positively expressed p53 protein.

G_1 phase.[23,24] Mean Ki-67 PR in the abnormal epithelium (inflammatory and dysplastic epithelium) was higher than that of normal epithelium.

It is well known that strong expression of oncogenes and loss of tumor-suppressor genes play important roles in tumogenesis. Alterations of the *p53* gene, one of the important suppressor genes, are the most common genetic changes detected in various human malignancies.[25,26] These changes play a critical role in the complex process of carcinogenesis. Shimaya et al[27] have reported that nuclear accumulation of p53 documented by immunohistochemistry may be an independent prognostic factor in esophageal carcinoma and have suggested that *p53* gene mutations may affect the growth rate of these tumors. In our study, p53 expression was demonstrated in the tumors of two patients (cases 2 and 3). Wang et al[28] frequently found p53 immunoreactivity and mutations of the *p53* gene in esophageal precancerous lesions, and Bennett et al[29] have suggested that *p53* gene mutation and protein accumulation in the esophagus may precede the morphologic changes seen during tumor development. In our studies, diffuse or localized abnormal epithelium was seen in all four patients, but p53 protein expression was not found in esophagitis and dysplasia lesions including normal epithelium.

The nuclear expression of Ki-67 was stronger in dysplasia and esophagitis lesions than in normal epithelium. This result suggests that the abnormal epithelium of achalasia is more proliferative than normal epithelium, therefore these cells may be more sensitive to carcinogens.

It is very difficult to cure achalasia patients with esophageal carcinoma because the majority of these patients have unresectable tumors. Marked weight loss, generalized deterioration, hematemesis and melena are unusual symptoms in achalasia patients and suggest malignant degeneration.[30,31] We should perform a thorough examination that includes esophagoscopy when achalasia patients have these symptoms. However, the widespread nature of esophagitis and/or dysplasia in these patients may make small cancers difficult to detect. We emphasize that cleaning of the esophagus followed by endoscopic iodine staining can identify early esophageal carcinomas. For curative surgery, we have to detect early stage cancer, however only one (case 3) of our patients was in this category.

In conclusion, the high proliferative activity of abnormal epithelium seen in achalasia may render this epithelium more sensitive to carcinogens.

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