

# Immunohistochemical expression of p63 in esophageal Barrett's adenocarcinoma

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**Abstract.** p63, which has recently been identified as a member of the p53 gene family, plays many important roles in human tissue functions. We examined p63 expression patterns in esophageal Barrett's adenocarcinoma, including early-stage cancers, as well as its clinicopathological significance. Immunoreactivity for p63 was detected in 45.8% (11/24) of the adenocarcinomas; all cases were focally positive. p63 was detected not only in the *in situ* carcinomatous components or intramucosal carcinomas, but also in the invasive carcinomatous regions of the p63-positive cases. Immunohistochemical expression was correlated with clinicopathological features; i.e., lymph node metastasis, lymphatic invasion and venous invasion were found in 72.7% ( $p<0.01$ ), 100% ( $p<0.05$ ) and 90.9% ( $p<0.05$ ) of the p63-positive cases, respectively, at a significantly high incidence compared with the p63-negative cases. We also analyzed the relationship between p63 and p53 expression. No diffuse expression of p63 was detected, although diffuse expression of p53 was detected in 50.0% (12/24) of the adenocarcinomas. The role of the p63 gene is thought to be different in esophageal Barrett's adenocarcinoma compared with esophageal squamous cell carcinoma. p63 immunoreactivity was most useful as a predictor of lymph node metastasis.

## Introduction

Barrett's mucosa is an inflammatory and reactive condition in which the normal squamous mucosa of the esophagus is

replaced by a metaplastic columnar epithelium (1,2). The disease may be caused by the esophageal reflux of digestive fluid, including gastric juice and/or bile, and is ordinarily lined by a gastric-type epithelium with or without intestinal metaplasia. Several studies have reported a high incidence of adenocarcinoma arising in Barrett's mucosa, and have analyzed and compared it to the morphological characteristics of esophageal adenocarcinoma (3,4). Previously, in esophageal Barrett's adenocarcinoma and non-neoplastic Barrett's mucosa around the carcinoma, we analyzed the distribution of mucin and CD10 and evaluated proliferative activity with a Ki-67 labeling index (LI) (5,6). It is suggested that Barrett's esophageal mucosa with intestinal metaplasia and a high Ki-67 LI is the premalignant lesion with the most impact (6).

p63, a homologue of the tumor-suppressor p53, is highly expressed in the basal or progenitor layers of squamous epithelia, such as the skin, esophagus and exocervix (7-9). In the stratified epithelia, p63 is essential for the proliferative potential of stem cells (10). In contrast, p63 expression is absent or restricted in colonic, mammary or prostatic epithelia (7). Recent studies have reported p63 expression predominantly in basal cell carcinomas, squamous cell carcinomas and transitional cell carcinomas, but not in adenocarcinomas, including gastric and colonic cancers (7,11-14). We have also analyzed the expression and histological distribution of p63 and p53 in esophageal squamous cell carcinomas, and reported that the results suggest the p63 gene plays a major role in the carcinogenesis of human esophageal squamous cells and in the growth of the carcinoma (15). However, the expression patterns of p63 in esophageal Barrett's adenocarcinoma have yet to be extensively analyzed.

In the present study, we examined the expression and histological distribution of p63 and p53 protein in esophageal Barrett's adenocarcinoma, including early-stage cancers, and discussed the clinicopathological significance of the disease.

## Materials and methods

**Esophageal tissue specimens.** All tissue specimens were obtained through surgical resections of esophageal Barrett's adenocarcinoma at Tokai University Hospital. Twenty-four

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cases of Barrett's adenocarcinoma were examined in this study. All were histopathologically defined as a columnar epithelium of the esophagus, i.e. Barrett's esophagus, using routine hematoxylin and eosin-stained sections. The adenocarcinomas were classified according to the TMN system (16). All esophageal specimens were rapidly fixed in 10% buffered formalin and routinely embedded in paraffin for 14-48 h for histological and immunohistochemical analysis. Tumor invasion, venous invasion and lymphatic invasion were examined on 4- $\mu$ m sections stained with hematoxylin and eosin.

**Immunohistochemical analysis.** Sections (4  $\mu$ m) were deparaffinized, and endogenous peroxidase activity was quenched by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min (17-20). Sections were autoclaved (ES-215, high-pressure steam sterilizer, Tomy, Japan) at 121°C for 4 min for antigen retrieval. Non-specific binding was blocked with normal sheep serum (Cosmo Bio Co. Ltd., Tokyo, Japan) in phosphate-buffered saline (PBS), and then slides were incubated with anti-p63 (clone 4A4, 1:1600; PharMingen International, San Diego, CA, USA) or anti-p53 monoclonal antibody (clone DO7, 1:20; Novo Castra, Newcastle, UK) overnight at 4°C. Immunoreactivity was detected using the peroxidase-labeled streptavidin-biotin (LSAB) method (Dako A/S, Copenhagen, Denmark) (21). Slides were subsequently incubated with biotinylated anti-mouse Ig(Fab)<sub>2</sub> antibody (Amersham International plc., Buckinghamshire, UK) at a dilution of 1:100 for 60 min, followed by detection using streptavidin-conjugated horseradish peroxidase.

**Assessment of p63 and p53 expression.** Tumor cells with nuclei containing brown immunoreaction products were counted as positive for p63 or p53 protein expression. Immunohistochemical expression patterns of p63 or p53 were divided into 4 groups as follows: (i) negative expression, (ii) sporadic expression with <5% of cancer cells positive for p63 or p53, (iii) focal expression with 5-50% of cancer cells positive for p63 or p53, and (iv) diffuse expression with >50% of cancer cells positive for p63 or p53. Cases of positive expression included (iii) focal expression and (iv) diffuse expression.

**Statistical analysis.** The  $\chi^2$  test and Fisher's exact test were used for comparisons between group frequencies. Statistical significance was defined as  $p < 0.05$ .

## Results

Immunoreactivity for p63 was detected in the nucleus but not in the cytoplasm of cells. The results of immunohistochemical analysis are summarized in Table I. Immunoreactivity in the cancer tissues was found in 11 (45.8%) of the 24 esophageal Barrett's adenocarcinomas examined; all cases were focally positive (Fig. 1). In this study, p63 expression was found not only in T1 tumors, but also in cases of invasive carcinoma. Immunohistochemical expression patterns of p63 and p53 are summarized in Table II. No diffuse expression of p63 was detected, although diffuse p53 expression was detected in 50.0% (12/24) of the adenocarcinomas.

Table I. p63 immunoreactivity and the clinicopathological features of esophageal Barrett's adenocarcinoma.

	p63 expression (%)		p-value
	Negative/ sporadic	Positive (focal)	
Esophageal cancer (n=24)	13 (54.2)	11 (45.8)	
Primary tumor			
T1	10 (66.7)	5 (33.3)	
T2	1 (50.0)	1 (50.0)	
T3/T4	2 (28.6)	5 (71.4)	
Differentiation			
Well	9 (56.3)	7 (43.7)	
Moderate	4 (50.0)	4 (50.0)	
Poor	0 (0.0)	0 (0.0)	
Regional lymph node metastasis			
(-)	11 (78.6)	3 (21.4)	
(+)	2 (20.0)	8 (80.0)	<0.01
Lymphatic invasion			
(-)	5 (100.0)	0 (0.0)	
(+)	8 (42.1)	11 (57.9)	<0.05
Venous invasion			
(-)	7 (87.5)	1 (12.5)	
(+)	6 (37.5)	10 (62.5)	<0.05

We analyzed the relationship between p63 expression and clinicopathological features, such as depth of tumor invasion, tumor differentiation and lymphatic/venous invasion (Table I). p63 was frequently detected in the cancer cells (45.8%, 11/24) regardless of the depth of tumor invasion or the differentiation of the tumor. Lymph node metastasis, lymphatic invasion and venous invasion were found in 72.7% (8/11) ( $p < 0.01$ ), 100% (11/11) ( $p < 0.05$ ) and 90.9% (10/11) ( $p < 0.05$ ) of the p63-positive cases, respectively, at a significantly high incidence compared with the p63-negative cases.

## Discussion

Using biopsy specimens, Hall *et al* detected the expression of p63,  $\Delta$ Np63 $\alpha$  and p53 in esophageal Barrett's adenocarcinomas (22). In the present study, we examined p63 and p53 expression immunohistochemically in esophageal Barrett's adenocarcinoma, and analyzed its clinicopathological significance using surgically resected specimens. Focally-positive p63 immunoreactivity was detected in 45.8% (11/24) of the adenocarcinomas, while no cases of diffuse expression were found in the present study. These patterns of p63 expression were similar to those of a study by Gedert *et al* (23). On the other hand, p63 immunoreactivity was detected at a high frequency in human esophageal squamous cell carcinomas, as shown in our previous study (15). p63 expression was found not only



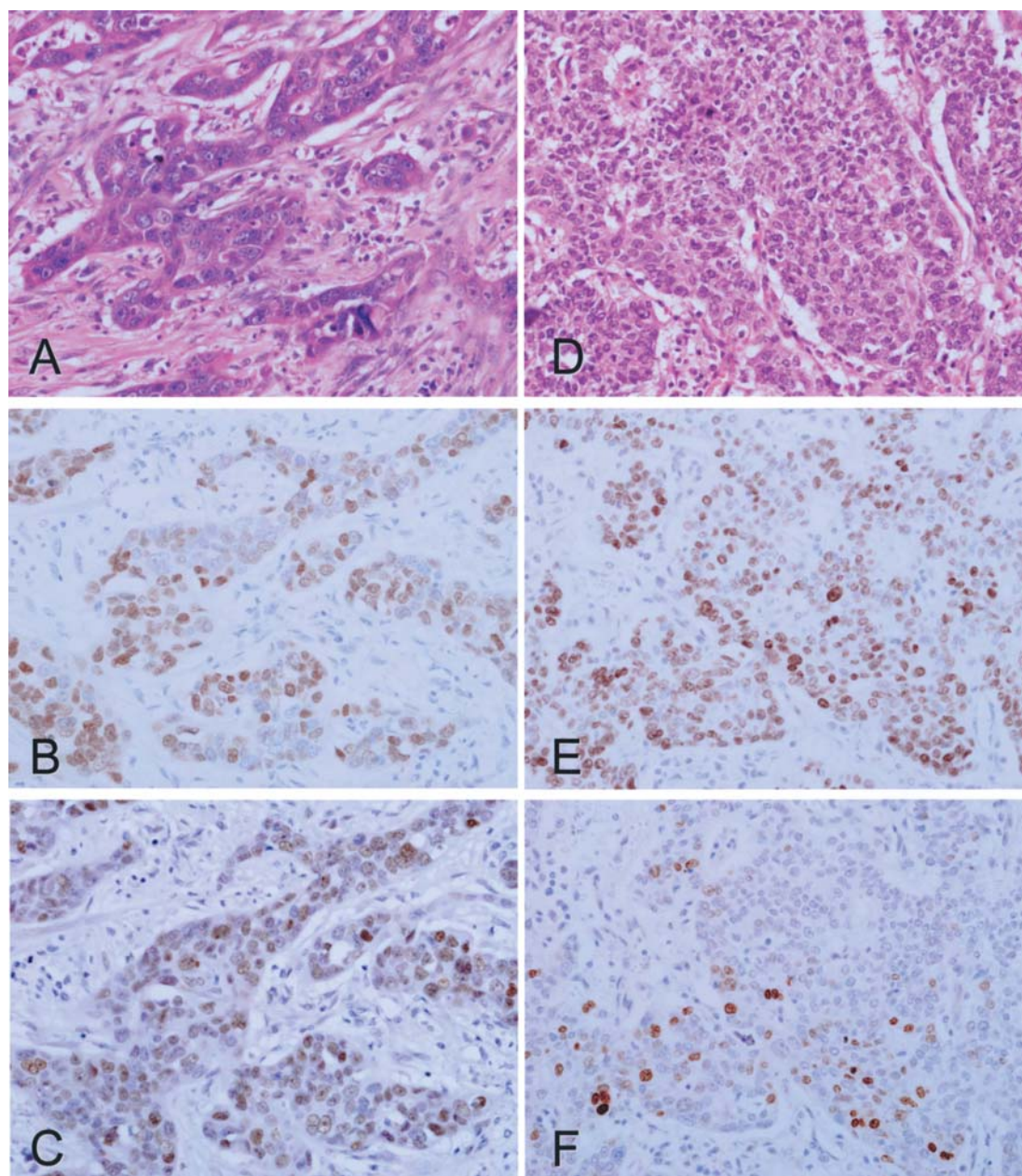


Figure 1. Microscopic findings of p63- and p53-positive esophageal Barrett's adenocarcinomas. (A) The carcinoma invaded the esophageal wall (hematoxylin and eosin). (B) Focal p63 immunoreactivity was detected in the nuclei of the carcinoma cells (the same case as A, LSAB method). (C) Diffuse p53 immunoreactivity was detected in the nuclei of the carcinoma cells (the same case as A, LSAB method). (D) The carcinoma invaded the esophageal wall (hematoxylin and eosin). (E) Focal p63 immunoreactivity was detected in the nuclei of the carcinoma cells (the same case as D, LSAB method). (F) Focal p53 immunoreactivity was detected in the nuclei of the carcinoma cells (the same case as D, LSAB method).

Table II. Immunoreactivity for p63 and p53 in esophageal Barrett's adenocarcinoma.

		p63		Total
		Negative/ sporadic	Positive (focal)	
p53	Negative/sporadic	4	2	6
	Positive (focal)	4	2	6
	Positive (diffuse)	5	7	12
	Total	13	11	24

in the T1 tumors, but also in invasive carcinomas. Lymph node metastasis, lymphatic invasion and venous invasion were frequently found in the p63-positive adenocarcinomas. This is the first report to analyze the relation between p63 expression and pathological factors, such as vascular invasion, in esophageal Barrett's adenocarcinoma using surgically resected specimens.

The p63 gene, located at 3q27-29, is a member of the p53 gene family. Its products have amino acid sequences similar to those of p53 gene products (9,24). Unlike p53, p63 gene products are divided into two groups: (i) an isotype with a transactivating domain (TA) in the N-terminal region and (ii) a  $\Delta N$  isotype ( $\Delta Np63$ ), which lacks the TA domain (9,25). In

tumor tissue, p63 is predominantly expressed in basal cell and squamous cell carcinomas, and not in adenocarcinomas, including those of the stomach and colon (7). A previous study reported that p63 was essential for embryological ectodermal differentiation, and that reduced expression of p63 had prognostic implications for patients with esophageal squamous cell carcinoma (26,27). A recent study reported that p63 is essential for the proliferative potential of stem cells in stratified epithelia (10). Each year, studies elucidate the varying roles of p63 in human tissue.

Previously, we clarified the significance of p63 in human esophageal squamous cell carcinoma (15). The majority of such carcinomas exhibited diffuse or focal p63 expression. Regardless of tumor invasion, differentiation, regional lymph node metastasis and lymphatic/venous invasion, p63 immunoreactivity was observed in almost all cases. Based on these results, we suggested that the p63 gene plays principal roles in the carcinogenesis of human esophageal squamous cells and the growth of the carcinoma. In the present study, we tried to clarify the significance of p63 in esophageal Barrett's adenocarcinoma. The established risk factors for this adenocarcinoma are Barrett's esophagus, gastro-esophageal reflux, and obesity (28,29). Some studies have described a relation between Barrett's esophagus and bile (30,31). Adenocarcinoma of the esophagus almost always arises as a consequence of the neoplastic progression of Barrett's esophagus (32). Numerous markers of genetic and molecular processes have been proposed in the carcinogenesis of Barrett's esophagus (33). Jenkins *et al* and Morales *et al* reviewed the genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma (34,35). On the other hand, conventional clinical risk factors for the development of Barrett's adenocarcinoma are neither sensitive nor specific enough for the classification of individuals at high risk, e.g., sex, age, race and body-mass index (31).

p63 was less frequently detected in esophageal adenocarcinomas (13) and, here, focal expression of p63 was found in 45.8% of the esophageal Barrett's adenocarcinomas. Lymph node metastasis, lymphatic invasion and venous invasion were found significantly more often in the p63-positive cases. These findings suggest that p63 expression plays a significant role in the lymph node metastasis, lymphatic invasion and venous invasion of esophageal Barrett's adenocarcinoma cells, rather than in tumorigenesis, depth of invasion and differentiation. In conclusion, p63 expression was shown to be more useful as a predictor of lymph node metastasis than of any other clinicopathological feature of esophageal Barrett's adenocarcinoma.

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