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Proliferative activity and p53 protein accumulation correlate with early invasive trend, and apoptosis correlates with differentiation grade in oesophageal squamous cell carcinomas

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Abstract Using oesophageal squamous cell carcinoma samples of both intramucosal and advanced types, proliferative activity (Ki-67 labelling index), p53 protein accumulation and apoptosis (in situ DNA nick end labelling) were assessed, and the relation of these values to progression or differentiation grade of tumours was analysed. In terms of proliferative activity and the proportion of positive cases with p53 accumulation, a statistically significant difference was demonstrated between intraepithelial carcinomas and intramucosal carcinomas with stromal invasion (17.2% vs 31.7% for the Ki-67 labelling index, and 23.5% vs 67.4% for the proportion of positive cases of p53 accumulation). Values for the latter were almost comparable to those of advanced carcinomas. Immunohistologically, Ki-67 positive, proliferating cells were distributed preferentially in the peripheral fronts of invading nests. Apoptotic cells were observed in the inner areas of the invading nests of the intramucosal carcinomas with stromal invasion and in more advanced lesions, but were rarely observed in the normal epithelium or intraepithelial carcinomas. Apoptotic cells were seen mainly around areas of keratinization, and the apoptotic cell index was higher in well and moderately differentiated types of advanced carcinomas than in the poorly differentiated type (2.59% vs 1.09%). An increase in proliferative activity and an accumulation of p53 protein are associated with the onset of early carcinomatous invasion, while apoptosis is closely linked with the differentiation grade of carcinoma cells.

Key words Oesophageal neoplasms · Squamous cell carcinoma · Proliferative activity · Ki-67 · p53 · Apoptosis

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Introduction

Squamous cell carcinomas of the oesophagus are relatively common in countries of eastern Asia, including China and Japan, and their prognosis is generally assumed to be poor. The recent remarkable progress in digestive endoscopy has contributed to the accuracy of diagnosis of oesophageal carcinomas, and popularization of Lugol's solution spray technique has improved the efficiency of detecting superficial carcinomas with invasion limited to the mucosa or submucosa [30]. In consequence, the number of patients with superficial oesophageal carcinoma has increased in recent years. Several reports [5, 17, 23, 24, 27] indicated that the 5-year survival rate was worse in superficial oesophageal carcinomas with submucosal invasion (33–69%) than in those without submucosal invasion (intramucosal carcinomas: 83–100%). Thus, intramucosal carcinoma is assumed to be a true "early carcinoma". Evaluation of the malignant potential of oesophageal carcinomas, especially in the early stages of tumour progression, is important to establish prognosis.

A number of reports have proposed various indices for the evaluation of malignant potential of tumour cells, and the proliferative activity of tumour cells is one of the most useful. The newly established monoclonal antibody MIB-1 reacts with the Ki-67 nuclear antigen, which is closely associated with cell proliferation and expressed in the G1, S, G2 and M phases of the cell cycle but not in quiescent cells. In addition, MIB-1 is useful not only for frozen but also for formalin-fixed, paraffin-embedded tissue sections [10, 12, 13, 19]. Nowadays, many investigators use the Ki-67 labelling index as an important characteristic of tumour cells that correlates with the prognosis of patients bearing various tumours [9, 11, 34, 40].

The oncosuppressor gene *p53* located on chromosome 17p13 encodes a nuclear protein that plays a crucial part in the regulation of cell proliferation [7, 21, 35]. Its functional inactivation through mutation or allelic deletion appears to be closely related to the development of many

tumours [26]. Mutations at exons 5, 6, 7, and 8 are frequently found in a variety of tumours, including oesophageal squamous cell carcinomas [14]. Mutant p53 proteins are more stable than their wild type counterparts, and their accumulation in tumour cells can be detected immunohistochemically. It has been reported that accumulation of p53 protein is related to a poor prognosis for the patients and high proliferative activity of tumour cells [15, 32].

When the biological behaviour of tumour cells is under scruting, it is necessary to consider not only their proliferative activity but also the rate of cell death, often by apoptosis. Cell death is a physiological process, which has a crucial role in embryogenesis and morphogenesis of various organs [18] and is also of direct significance to cell turnover in neoplasia [39]. The in situ DNA nick end labelling method is useful for detection of apoptotic cells in paraffin-embedded sections [8].

In advanced cases of oesophageal squamous cell carcinoma [25, 41], information is available about the relation of various features of tumour cells (proliferative activity, accumulation of p53 protein and apoptosis) to prognosis, and also to progression and to differentiation of tumour cells. However, these features of tumour cells have not been fully examined in intramucosal oesophageal carcinomas, especially in terms of their early invasive tendencies.

In this communication, we focus on cell proliferation and apoptosis in intramucosal oesophageal carcinomas. By comparing these features with those of more advanced cases, we have attempted to reveal a correlation between cell proliferation and death and tumour progression or differentiation of oesophageal squamous cell carcinomas.

Materials and methods

One hundred and thirteen surgical specimens of oesophageal squamous cell carcinomas were selected from the patient files of the Department of Pathology, Tokyo Medical and Dental University Hospital and its associated hospitals from 1989 to 1994: 60 were from intramucosal carcinomas and 53 were from advanced lesions with invasion of the proper muscular layer or adventitia. Neither pre-operative radiation nor chemotherapy had been performed in any of the cases examined. Twenty-six patients with intramucosal carcinoma underwent endoscopical mucosal resection, and the remaining 87 patients, oesophagectomy and lymph node resection. All the intramucosal carcinomas were diagnosed as primary lesions, and synchronous lesions found incidentally co-existing with the more advanced tumours were excluded from the present study, because effects of advanced tumours on the proliferative activity of smaller lesions could not be ruled out. All the resected specimens were fixed in buffered formalin solution, sliced serially at 3–5 mm and routinely processed for paraffin blocks. Endoscopically resected samples had been fixed overnight, and surgically resected samples had been fixed for 2 or 3 days; however, this difference in handling did not influence the results of immunohistochemistry and in situ DNA nick end labelling.

Sixty intramucosal carcinoma cases were classified into four stages, *ep*, *mm*1, *mm*2 and *mm*3, according to the degree of tumour invasion (Fig. 1). The criteria for each stage are as follows: *ep* means intraepithelial carcinoma in the strict sense, with a straight epithelial–stromal border (whole layers of the epithelium are replaced by atypical cells, the border with surrounding normal epithelium is clear, and rete ridge elongation is rarely observed). *mm*1 means carcinoma with early stromal invasion (the epithelial–stromal border is irregular, and the rete ridge shows elongation into the propria mucosae, but budding of the elongated rete ridge or formation of small invading nests are rarely observed). *mm*2 means carcinoma with overt stromal invasion demonstrating remarkable budding of the elongated rete ridge and formation of small invading nests, but no encroachment into the muscularis mucosae. *mm*3 means carcinoma with muscularis mucosal involvement but no invasion of the submucosa.

Each case was classified into well, moderately, and poorly differentiated types according to the histological classification of the World Health Organization [38]. Of 60 intramucosal carcinomas, only 3 *mm*3 cases had lymph node metastases. Up to the present, at least 24 months after the last operation, no patients with intramucosal carcinomas have died of recurrence after the operation.

Monoclonal antibodies used in the present study were against Ki-67 (MIB-1, Immunotech) [13] and p53 (DO7, Novocastra) [2], and the streptavidin–biotin method was used for colour visualization. DO7 antibody detects both wild and mutant forms of the p53 protein. Monoclonal antibodies against Ki-67 and p53 proved useful for paraffin sections.

Paraffin-embedded sections were laid on poly-L-lysine-coated slides, and deparaffinized. Digestion by 0.1% trypsin for 10 min at 37°C and microwave heating in 5% urea solution for 10 min at 95°C were required when antibody against Ki-67 (MIB-1) was applied. Microwave heating in 20% zinc sulfate solution for 10 min at 95°C was required when antibody against p53 (DO7) was applied. Sections were incubated in methanol containing 0.3% (v/v) H_2O_2, washed in PBS, and incubated in normal rabbit serum followed by reaction with primary antibodies for 2 h at room temperature in humidified chambers. After washing with PBS, the sections were reacted with biotinylated anti-mouse immunoglobulin, followed by incubation with horseradish peroxidase labelled streptavidin (Nichirei). After three additional washes, peroxidase was developed with 0.02% diaminobenzidine (Sigma) at pH 7.6 in 0.05 M Tris buffer plus 0.015% H_2O_2. The slides were counterstained with haematoxylin or methyl green. Sections incubated with normal mouse serum in place of the primary antibody were used as negative controls. Sections from a case of colon cancer were used as positive controls for immunostaining of p53 and Ki-67. In this positive control case, mutation of the p53 gene (codon 248; Arg → Gln) was confirmed by SSCP and direct sequencing analysis.

In each case, the Ki-67 labelling index (LI) of the lesion was calculated as the percentage of positive cells divided by the total number of cells examined in all fields. The immunostained sections were evaluated blindly by two experienced observers to avoid bias. Fields were randomly selected from the area where carcinoma invasion was most marked and carcinoma nests did not show massive necrosis, and approximately 2,000 nuclei were counted in each case. Stromal cells positive for Ki-67 were carefully excluded from the counting process. In intramucosal carcinoma cases, invasive lesions were limited to a small area in general. We obtained good reproducibility of the data, and inter-observer variations were minimal. In advanced cases, proliferative activity varied between different areas of the same invasive lesion, especially in the well differentiated type, but by counting approximately 2,000 nuclei in randomly selected fields, we made the inter- and intra-observer variations in labelling index small enough (less than 5%) for their effect on interpretation to be negligible. Intramucosal oesophageal carcinomas have two different growth directions: one is the downward invasive lesion and the other is the intraepithelial spread (*ep* lesion) adjacent to the invasive change. Intraepithelial neoplastic lesions were observed in 31 cases (72%) of *mm* carcinomas. Ki-67 LI was also assessed in intraepithelial neoplastic lesions.

The extent of the p53 nuclear reactivity was classified into four grades: (–), no reactivity; (1+), focal presence of positive cells (1–10% tumour cells); (2+), heterogeneous nuclear reactivity (10–50% tumour cells); (3+), homogeneous intense nuclear reac-

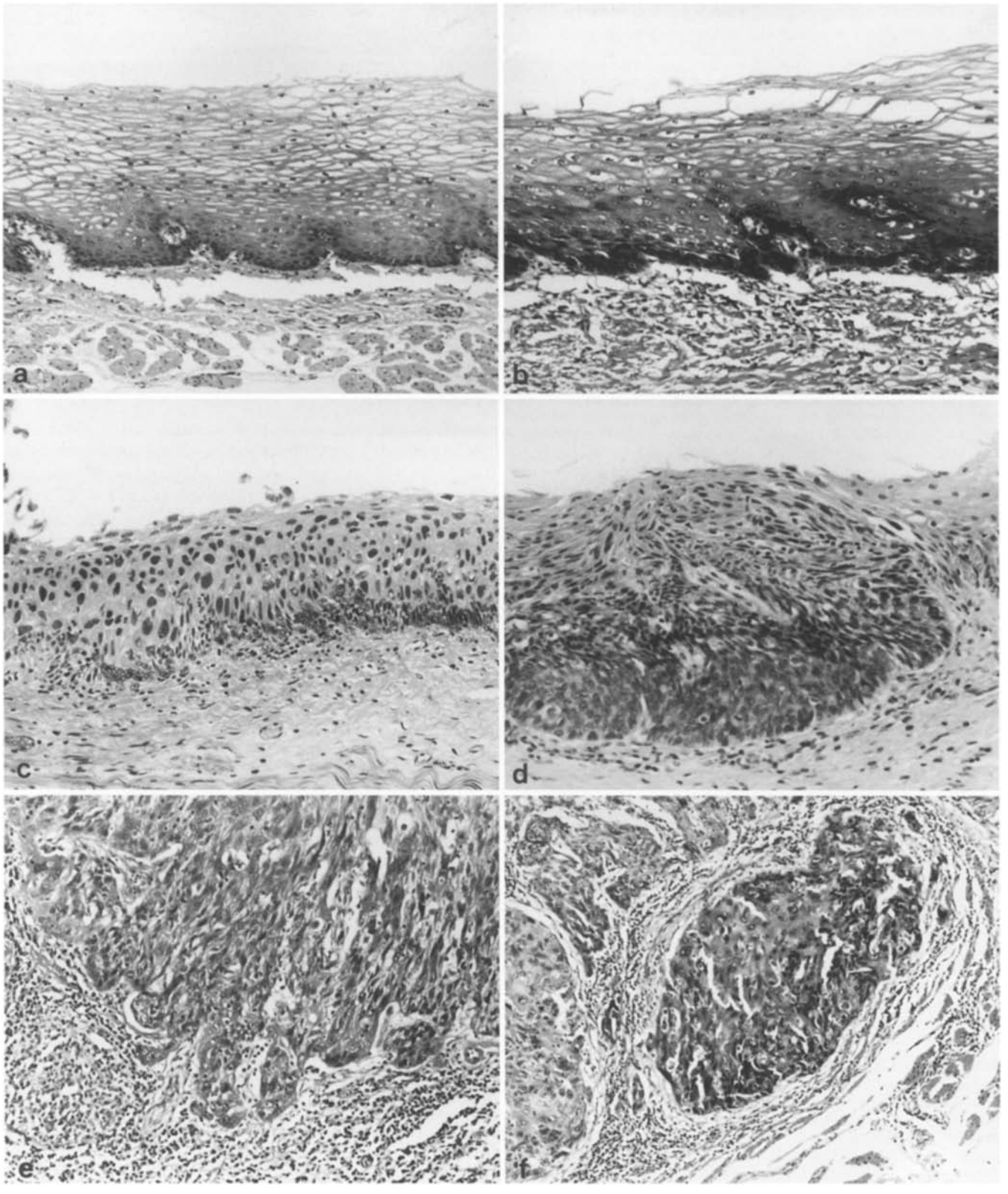

Fig. 1a–f Histological features of normal epithelium, dysplasia, and typical intramucosal carcinomas. **a** Normal epithelium. HE, ×33. **b** Dysplasia, incidentally observed around the intramucosal carcinoma. Atypical cells are distributed in the basal and parabasal layers of the epithelium. HE, ×40. **c** *ep* carcinoma. Atypical cells occupy the whole thickness of the epithelium, but the epithelial–stromal border is almost straight. HE, ×33. **d** *mm*1 carcinoma. The epithelium shows thickening and elongation of the rete ridge, but budding and formation of small invading nests are not observed. HE, ×50. **e** *mm*2 carcinoma. The elongated rete ridge shows irregular budding into the stroma, resulting in the formation of small invading nests. HE, ×25. **f** *mm*3 carcinoma. The carcinoma is invading the muscularis mucosae, but not the submucosa. HE, ×25

tivity (50–100% tumour cells). p53 nuclear reactivity in the intraepithelial neoplastic lesions was also estimated in the same manner.

In situ DNA nick end labelling was carried out based on the previously described method [3]. Paraffin embedded sections mounted on coated slides were deparaffinized, hydrated, and treated with proteinase K for 30 min at 37°C in a humidified chamber (Boehringer Mannheim; 10 μg/ml in 20 mM Tris and 2 mM $CaCl_2$, pH 7.4), followed by three washes in Tris buffer (100 mM Tris and 150 mM NaCl, pH 7.5). DNA 3'-end labelling with digoxygenin-ddUTP (dig-ddUTP) was performed after incubation for 10 min in terminal deoxynucleotidyl transferase (TdT) buffer (200 mM potassium cacodylate, 25 mM Tris, 0.25 mg/ml BSA, and 5 mM $CoCl_2$, pH 6.6) at room temperature. TdT(1 U/μl; Boehringer Mannheim), dig-ddUTP (5 μM; Boehringer Mannheim) and ddATP (45 μM; Boehringer Mannheim) were added in buffer and incubated at 37°C in a humidified chamber for 1 h. After three washes in Tris buffer, the sections were incubated with blocking buffer (0.5% wt/vol blocking reagent; Boehringer Mannheim, in Tris buffer) for 30 min at room temperature before the addition of antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim). After incubation with antibody (1:500) at room temperature for 30 min, the slides were washed three times in Tris buffer and finally equilibrated in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) before the addition of substrate (337.5 μg/ml nitroblue tetrazolium and 175 μg/ml 5-bromo-4-chloro-3-inodyl-phosphate; Boehringer Mannheim) for alkaline phosphatase. After 15 min to 1 h in the dark, the colour reaction was terminated with 10 mM Tris and 1 mM EDTA, pH 8. Sections were counterstained with haematoxylin. For the positive control, sections were treated with 0.7 μg/ml DNase I (Stratagene) in potassium cacodylate buffer (pH 7.2) for 10 min before treatment with TdT reaction solution. Negative controls included omission of TdT or dig-ddUTP from the reaction solution.

The apoptotic cell index was calculated as the percentage of positive cells divided by the total number of cells examined in all fields examined. Fields were randomly selected from the areas where invasion was most marked and carcinoma nests did not show massive necrosis. Approximately 2,000 nuclei were counted in each case. In advanced cases, the apoptotic cell index also varied between different areas of the same invasive lesion, especially in the well differentiated type, but as before, by counting approximately 2,000 nuclei in randomly selected fields, we reduced the inter- and intra-observer variations to less than 0.5%. To demonstrate the distribution pattern of proliferating cells and apoptotic cells, double staining for Ki-67 immunostaining and in situ DNA nick end labelling was also performed in the typical well-differentiated carcinoma cases.

To evaluate the significance of difference in the Ki-67 LI or apoptotic cell index between the two groups, the Mann–Whitney U-test was used. To evaluate the significance of correlation of p53 accumulation with tumour progression or differentiation, Fisher's exact test was used.

Results

Figure 1 demonstrates histological examples ranging from normal epithelium to *mm*3 carcinoma, which were examined in the present study. Data on the relationship between Ki-67 LI and the depth of tumour invasion are summarized in Table 1. Ki-67 LI of *ep* carcinomas was 17.2±5.76% (mean±sd), and significantly lower ($P<0.0001$) than the value (31.7±10.5%) for *mm*1 to *mm*3 combined (*mm* carcinomas). There was no significant difference in Ki-67 LI among *mm*1, *mm*2, and *mm*3, or between *mm* and advanced carcinomas. Ki-67 LI of the intraepithelial neoplastic lesions (*ep* lesions) around the invasive area of *mm* carcinomas was 18.4±5.05%, which was almost comparable to that of *ep* carcinomas.

Ki-67 positive cells were seen sporadically in the parabasal layer of normal oesophageal epithelium (Fig. 2a). In dysplasia incidentally observed around the intramucosal carcinoma, positive cells were distributed among the atypical cells of the basal and parabasal layers (Fig. 2c). In the *ep* carcinomas, they were scattered through whole layers of epithelium (Fig. 2e). In *mm*1, *mm*2, and *mm*3 carcinomas Ki-67-positive cells were increased in number and were preferentially distributed in the peripheral layers of the elongated rete ridge or the peripheral fronts of invading nests (Fig. 2g).

Fig. 2a–h Immunostaining of Ki-67 and p53 protein in normal epithelium, dysplasia and intramucosal carcinoma. **a** Ki-67 immunostaining in normal epithelium. Positive cells are scattered in the parabasal layer of the epithelium. ×50. **b** p53 immunostaining in normal epithelium. Note the negative staining. ×40. **c** Ki-67 immunostaining in dysplasia. Positive cells are distributed among the atypical cells of the basal and parabasal layers. ×40. **d** p53 immunostaining in dysplasia. This typical lesion shows negative staining. ×40. **e** Ki-67 immunostaining in an *ep* carcinoma. Positive cells are distributed throughout the epithelium. ×66. **f** p53 immunostaining in an *ep* carcinoma. Positive cells are distributed through the layers, as in the case of Ki-67 immunostaining. ×66. **g** Ki-67 immunostaining in an *mm*1 carcinoma. Positive cells are preferentially distributed in the peripheral layers, invading fronts of the elongated rete ridge. ×66. **h** p53 immunostaining in an *mm*1 carcinoma. Positive cells are distributed preferentially in the peripheral layers, invading fronts of the elongated rete ridge, as in the case of Ki-67 immunostaining. ×66

Table 1 Ki-67 labelling index and p53 accumulation in oesophageal carcinomas, and their relationship to tumour progression (formalin-fixed, paraffin-embedded sections)

Degree of carcinoma invasion	Ki-67 labelling index, (%) positive cells (mean±SD)	Cases positive for p53 accumulation (%)
Intramucosal carcinoma (*n*=60)		
ep (*n*=17)	17.2±5.76*	23.5** (1+, 17.6; 2+, 5.88; 3+, 0)
mm carcinoma combined *mm*1 to *mm*3 (*n*=43)	31.7±10.5*	67.4** (1+, 25.6; 2+, 30.2; 3+, 11.6)
*mm*1 (*n*=13)	34.6±12.3	69.2 (1+, 38.5; 2+, 23.1; 3+, 7.69)
*mm*2 (*n*=10)	29.4±10.1	70.0 (1+, 30.0; 2+, 30.0; 3+, 10.0)
*mm*3 (*n*=20)	31.0±10.3	65.0 (1+, 20.0; 2+, 40.0; 3+, 5.00)
Intraepithelial neoplastic lesion (*ep* lesion) of *mm* carcinoma (*n*=31)	18.4±5.05	48.4 (1+, 29.0; 2+, 12.9; 3+, 6.45)
Advanced carcinoma (*n*=53)	31.2±6.67	67.9 (1+, 13.2; 2+, 24.5; 3+, 30.2)

*$P<0.0001$; **$P<0.05$

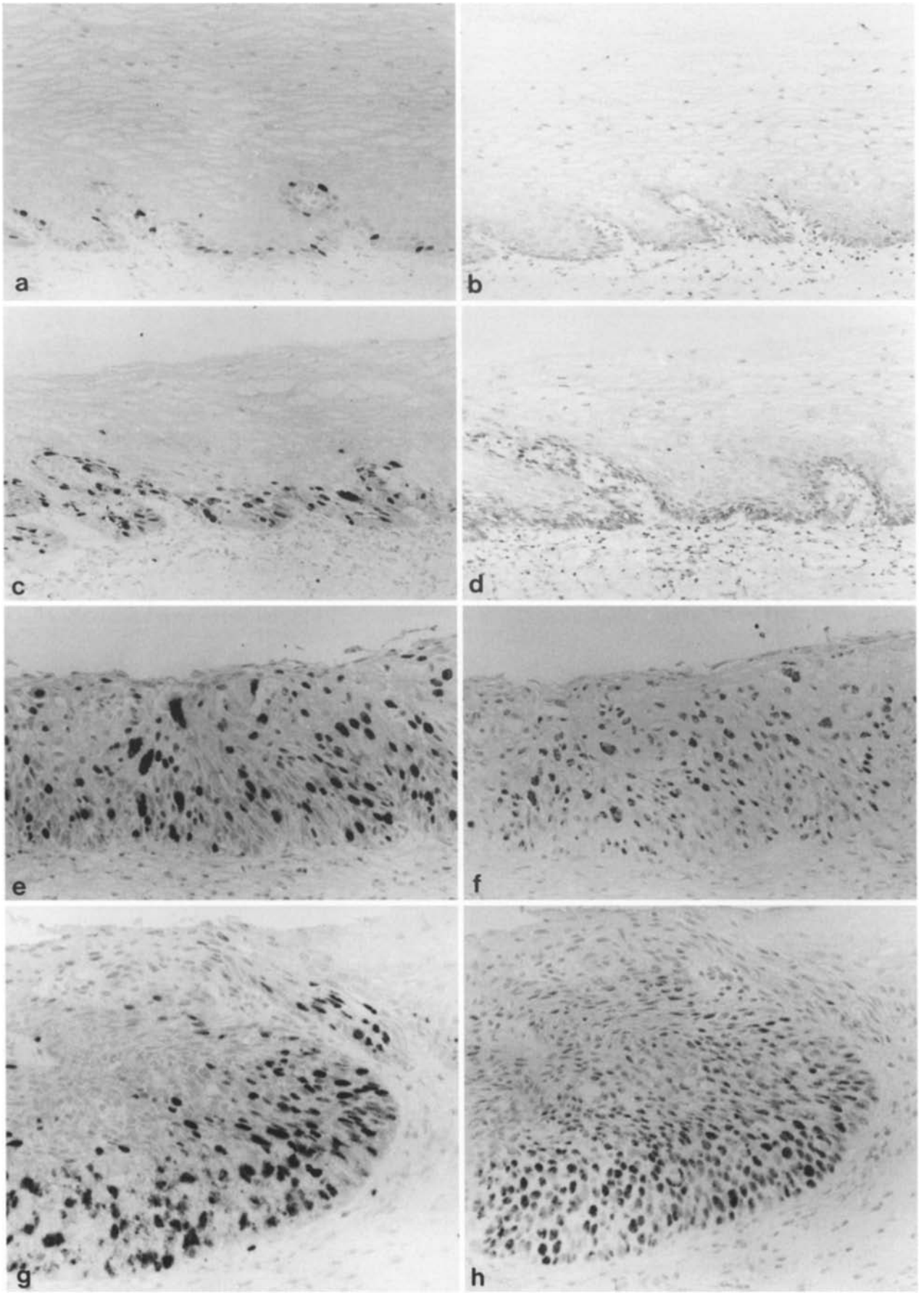
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Table 2 Ki-67 labelling index, p53 accumulation and apoptotic cell index in oesophageal carcinomas, and their relationships to tumour progression and differentiation

Types of carcinoma	Ki-67 labelling index positive cells (%) (mean ± SD)	p53 accumulation positive cases (%)	Apoptotic cell index positive cells (%) (mean ± SD)
Intramucosal carcinoma (*mm*1 to *mm*3)			
Well and moderately differentiated types (n=16)	31.9±9.78	62.5	1.84±1.29*
Poorly differentiated type (n=27)	31.6±11.6	70.4	1.12±0.68*
Advanced carcinoma			
Well and moderately differentiated types (n=35)	31.6±6.57	71.4	2.59±1.63**
Pooly differentiated type (n=18)	30.3±6.96	61.1	1.09±0.83**

*P>0.05; **P<0.01,

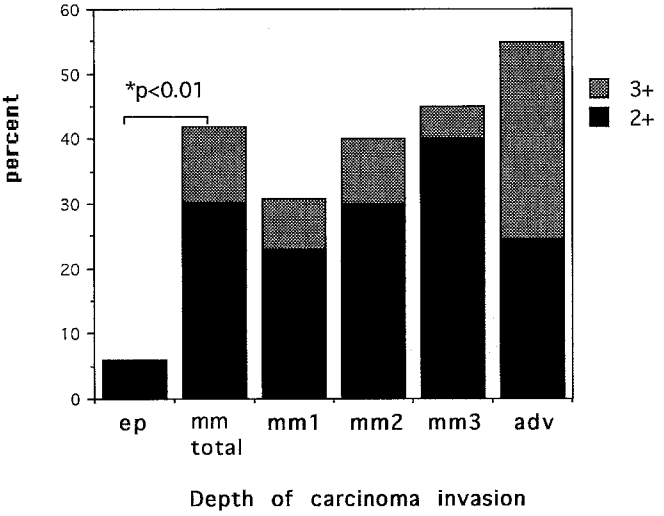

Fig. 3 Relationship between progression assessed as carcinoma invasion, and high expression of p53 protein. Cases with p53 accumulation increase in line with the degree of carcinoma invasion, statistically significant differences being observed between *ep* and *mm*1 to *mm*3 combined (P<0.01)

No significant correlation was observed between Ki-67 LI and the degree of differentiation of oesophageal carcinoma in either *mm* or advanced carcinoma categories (Table 2).

p53 protein accumulation was demonstrated in 4 out of 17 *ep* cases (23.5%), and 29 out of 43 *mm* cases (67.4%), the difference being significant (P<0.05; Table 1). There were no significant differences in the proportions of positive cases among *mm*1, *mm*2 and *mm*3, or between *mm* and advanced carcinomas (Table 1). p53 protein accumulation in areas of the intraepithelial neoplastic lesions (*ep* lesions) was demonstrated in 15 out of 31 cases (48.4%), which was intermediate between *ep* and *mm* carcinoma values, with no significant differences being obtained.

Since intense staining of p53 protein has been reported to correlate directly with the presence of a *p53* gene mutations [6], we next focused on cases of intense staining of p53 protein from grade (2+) to (3+). In this respect, the positive rate of intense expression of p53 increased in line with the depth of carcinoma invasion: the values for *ep*, *mm*1, *mm*2, *mm*3 and advanced carcinoma were 5.88, 30.8, 40.0, 45.0 and 54.7, respectively (Fig. 3). Statistically significant differences were demonstrated between *ep*and combined *mm*1 to *mm*3 (P<0.01), as for KI-67 LI.

Regarding the relation of p53 protein accumulation to the degree of tumour differentiation, no significant difference was observed between relatively well-differentiated (well and moderately differentiated types, keratinizing type) and poorly differentiated types (non-keratinizing type) of both *mm* and advanced carcinomas (Table 2).

Immunohistological testing of the distribution of p53-positive cells showed they were absent from all normal epithelium around any carcinoma. Dysplasia found incidentally around intramucosal carcinomas was also negative in the present series. In the *ep* cases positive cells were scattered in all layers of the epithelium (Fig. 2f), while in *mm* carcinomas they were preferentially located in the peripheral layers of the elongated rate ridge, or peripheral fronts of invading nests (Fig. 2h), which means quite a similar distribution to that of Ki-67 positive cells.

In both *ep* carcinomas and the intraepithelial neoplastic lesions (*ep* lesions) of *mm* carcinomas, apoptotic cells were rarely observed by in situ DNA nick end labelling, and it was almost impossible to calculate the apoptotic cell index (less than 0.1%). However, the frequency of apoptosis was significantly increased both in *mm* carcinomas and in advanced lesions, with apoptotic cell indices of more than 1.0% (Table 2). The apoptotic cell index was higher in relatively well-differentiated lesions (keratinizing type) than in their poorly differentiated counterparts (non-keratinizing type) in both *mm* carcinomas (1.84±1.29% vs 1.12±0.68%) and more advanced (2.59±1.63% vs 1.09±0.83%) categories, statistical significance being obtained for the latter comparison (P<0.01). These results were in contrast to the finding of almost the same proliferative activity assessed by Ki-67 LI, regardless of the degree of differentiation (Table 2).

In both *mm* and advanced carcinomas, there was no significant correlation and no inverse correlation between the Ki-67 LI and the apoptotic cell index (r = –0.063 in *mm* carcinomas and r = –0.41 in advanced lesions).

On haematoxylin/eosin-stained sections, apoptotic cells were recognized by pyknotic nuclei or nuclear frag-

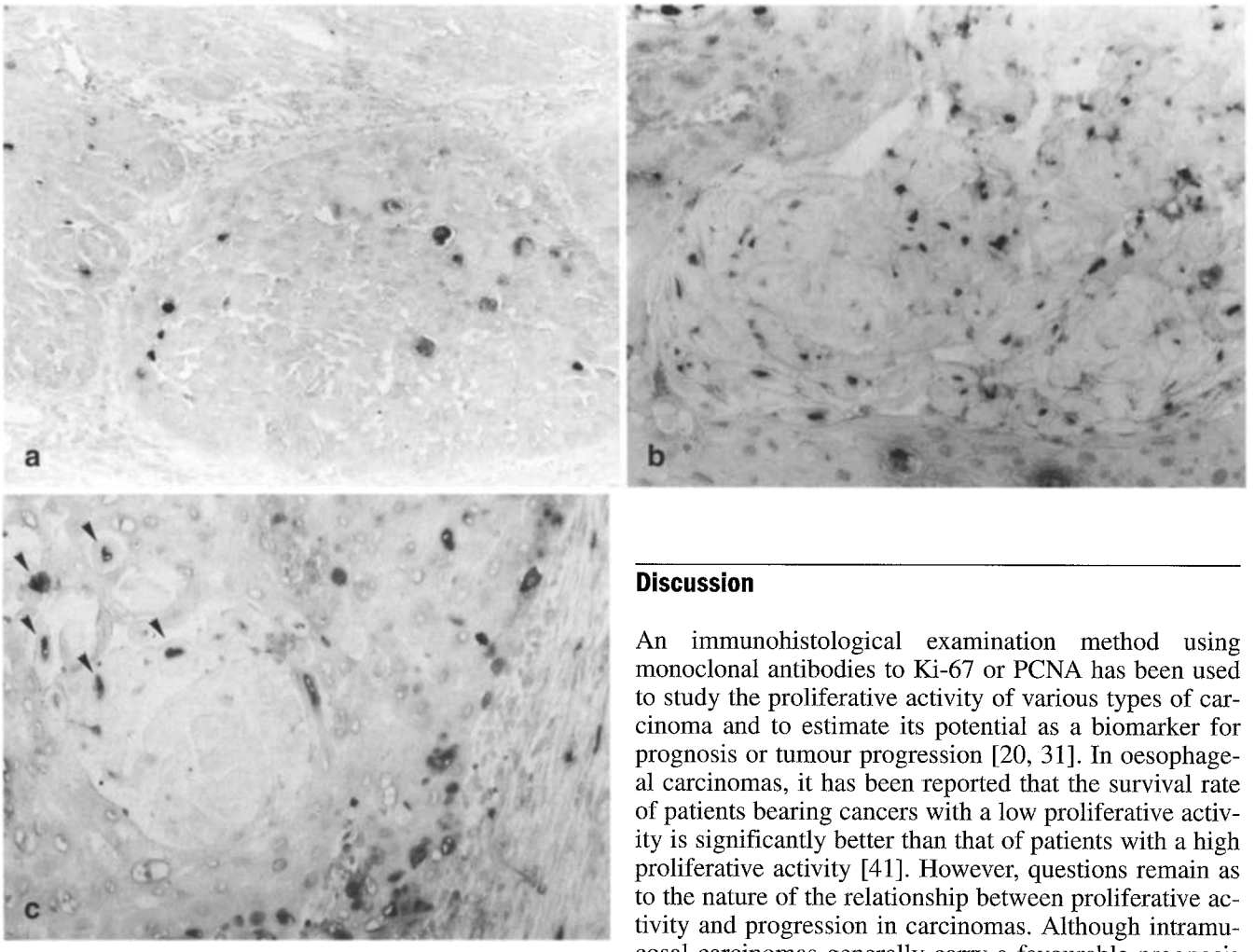

Fig. 4a–c Apoptosis of the intramucosal and advanced oesophageal carcinomas. **a** In situ DNA nick end labelling of the intramucosal case. Apoptotic cells with darkly stained nuclei are distributed sporadically in the inner area of the invading nest. ×33. **b** In situ DNA nick end labelling of the well-differentiated, advanced carcinoma case. Apoptotic cells are frequently observed in the inner areas of the invading nest, especially around the keratinizing lesion. ×33. **c** Double staining of in situ DNA nick end labelling and Ki-67 immunostaining (same case as **b**). Apoptotic cells (dark blue nuclei with *arrowheads*, *left upper corner*) are located in the inner area of the nest, and Ki-67-positive cells (brown nuclei, *right lower corner*) are shown distributed in the peripheral fronts. ×50

ments with vacuole formation, the usual characteristics of an apoptotic body. Most positive cells with in situ DNA nick end labelling had such features, but some lacked pyknosis. Mitotic cells and neutrophils in carcinoma nests were rarely stained positive by this method. Labelled apoptotic cells were scattered mainly in the inner areas of the elongated rete ridge and the invading nests (Fig. 4a), and this distribution was quite different from that of Ki-67 positive cells or p53-positive cells (Fig. 4c). It was interesting to note that apoptotic tumour cells were frequently seen around areas of keratinization, especially in cases of the well-differentiated type (Fig. 4b,c).

Discussion

An immunohistological examination method using monoclonal antibodies to Ki-67 or PCNA has been used to study the proliferative activity of various types of carcinoma and to estimate its potential as a biomarker for prognosis or tumour progression [20, 31]. In oesophageal carcinomas, it has been reported that the survival rate of patients bearing cancers with a low proliferative activity is significantly better than that of patients with a high proliferative activity [41]. However, questions remain as to the nature of the relationship between proliferative activity and progression in carcinomas. Although intramucosal carcinomas generally carry a favourable prognosis regardless of whether or not stromal invasion is evident [5, 17, 23, 24, 27], a difference in proliferative activity between *ep* carcinomas and *mm* carcinomas, in which an early invasive trend such as irregular epithelial–stromal border and elongation of rete ridges is shown, was revealed in the present study. Ki-67 LI was found to be significantly lower in *ep* carcinomas than *mm* carcinomas (17.2% vs 31.7%). Nowadays, endoscopical mucosal resection is increasingly performed for patients with intramucosal oesophageal carcinomas [4], on the basis of the data indicating that frequency of lymph node metastasis or vascular permeation is low in intramucosal carcinomas. However, the present results showing that proliferative activity of *mm* carcinomas is already as high as that of advanced carcinomas (31.2%) shows that complete removal of the tumour and careful follow-up are indispensable when endoscopical mucosal resection is performed.

Oesophageal squamous cell carcinomas have two different growth directions: downward invasion and intraepithelial neoplastic extension (*ep* lesion) adjacent to the invasive component. Although it is uncertain whether the intraepithelial neoplastic lesion represents intraepithelial spread or a pre-invasive lesion, it is notable that the proliferative activity assessed by Ki-67 LI was different be-

tween two components, with higher values for the downward invasion. Increased proliferative activity in the downward invading lesion, especially in the peripheral fronts of invading nests, might be related to the destruction of basement membrane and budding of carcinoma nests. Carcinomatous invasion is usually accompanied by destruction of basement membrane and remodelling of extracellular matrix [33]. Immunohistochemical analysis of basement membrane components has revealed that most oesophageal *ep* carcinomas show a well-preserved, continuous structure, while more than 50% of *mm* carcinomas have some defects [1]. Further studies on the changes in basement membrane structure and the expression of proteolytic enzymes, such as matrix metalloproteinases, in intramucosal oesophageal carcinomas will be valuable.

p53 is one of the most significant oncosuppressor genes implicated in the development of various malignant tumours. In the present study of advanced oesophageal carcinomas, we detected p53 accumulation in 67.9% of cases by our immunohistological method. These values were quite comparable to 45–85% reported by previous investigators [29, 36, 37]. We found that the value increased in line with progression of carcinoma invasion, and there was a significant difference between *ep* and *mm* carcinomas in the proportion of positive cases of p53 accumulation (23.5% vs 67.4%), as for the proliferative activity (see above). In a previous report from northern China, where the incidence of oesophageal carcinoma is extremely high, p53 accumulation was demonstrated in more than 70% of dysplasia cases. Although mutations of the *p53* gene were not examined in that report, alteration of the *p53* gene was considered to be an early event occurring in precancerous lesions [37]. In the present study, dysplastic lesions were not collected systematically, and the number of these lesions found incidentally was small. However, the present results demonstrated that p53 protein accumulation also affected the progression from *ep* to *mm*1, that is the onset of early invasion, in Japanese subjects. Mutations of the *p53* gene should be examined by molecular methods in cases of dysplasia in future.

Apoptosis is another important factor in determining progression of cancer. In gastric adenocarcinomas, it has been reported that advanced lesions show a higher frequency of apoptotic cells than their early counterparts [28]. However, the relationship between apoptosis and the differentiation grade of carcinoma cells remains controversial [16, 28]. In advanced cases of oesophageal carcinoma, it was reported that the apoptotic cell index was significantly higher in the keratinizing than in the non-keratinizing type [25]. In the present study, we confirmed the close correlation between apoptosis and differentiation grade of cancer cells in advanced carcinomas. In *mm* carcinoma cases, there was also a tendency for the apoptotic cell index to be higher in the keratinizing than in the non-keratinizing type, but the difference did not reach statistical significance. In the normal epidermis of the skin, where keratinization is observed in the superficial layers, apoptosis is reported to occur in the granular layers preparing for terminal differentiation [22]. In the present study, apoptosis was rarely seen in normal oesophageal epithelium and *ep* carcinomas, in which keratinization was not generally observed. Since apoptosis was directly linked with keratinization in the invading nests, a common mechanism might be present for induction of apoptosis and keratinization in both normal epidermis and invading nests of keratinizing carcinomas, especially in advanced cases. The apoptotic cell index of *mm* and more advanced carcinomas, especially of well-differentiated and moderately differentiated (keratinizing) types, increases together with the increase in proliferative activity in comparison with those of *ep* carcinomas. Although an increase of apoptosis is considered to be a disadvantage for the development of cancer, the degree of increased proliferative activity probably overcomes the disadvantage of *mm* and more advanced carcinomas.

In summary, an increase in proliferative activity and in p53 accumulation are associated with an early invasive tendency of oesophageal carcinomas. Apoptosis is also increased in early invasive (*mm*) carcinomas, and is also closely linked with the differentiation grade of cancer cells. Further studies of molecular regulation of cell cycle and induction mechanisms of apoptosis are necessary to detail the process of development and differentiation of oesophageal squamous cell carcinomas.

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