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Expression of the cyclin dependent kinase inhibitor p21$^{WAF1/CIP1}$ in oesophageal squamous cell carcinomas

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Abstract To elucidate the role of CDK inhibitor p21$^{WAF1/CIP1}$ in human oesophageal squamous cell carcinomas, we examined its expression immunohistochemically using surgically resected tissues from 25 patients, and have analyzed the relationship with alteration of p53 gene (F-SSCP analysis), proliferative activity (Ki-67 labelling index), frequency of apoptosis (in situ DNA nick end labelling), and degree of differentiation. P21 expression was observed in 11 cases (44%) with a percentage of positive cells ranging between 1% and 10%. Of the 25 cases, 4 cases showed >5% of positive cells. As for the relationship with p53 gene, all 7 p53-mutation positive cases were negative for p21 expression, whereas 11 out of 18 mutation negative cases showed positive for p21 expression. As for the relationship with degree of tumour differentiation, 6 out of 8 well differentiated type cases showed positive for p21 expression. By contrast, all 8 cases of poorly differentiated type were negative for p21 expression. Frequency of apoptotic cells was significantly higher in p21 positive cases than negative cases although Ki-67 labelling index was almost the same regardless of the expression of p21. P21 expressing cells were distributed mainly in the middle layers of the invading nests, especially around the keratinization, which was almost similar to the distribution of apoptotic cells. Our results suggest that expression of p21 in human oesophageal squamous cell carcinomas is induced by a p53-dependent pathway and affects apoptosis and differentiation of carcinoma cells.

Key words Oesophageal neoplasms · Cyclin-dependent kinase inhibitor · *p21* · *p53* · Apoptosis

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Introduction

Squamous cell carcinomas of the oesophagus are relatively common in east Asian countries, such as China and Japan. The prognosis of such tumours is poor, and elucidation of the molecular mechanisms of tumour growth and the establishment of informative biomarkers are thus under intensive investigation [26, 27, 40]. Previously, we have examined cell proliferation, accumulation of p53 protein, and apoptosis in these carcinomas and found that an increase of proliferative activity and accumulation of p53 proteins correlate with an early invasive trend and apoptosis correlates with differentiation of the carcinoma cells [27].

The tumour suppressor gene *p53* is considered to have a crucial role in the regulation of cell proliferation [12, 20, 24, 35]. Its functional inactivation through mutation or allelic deletion appears to be closely related to the development of many tumours, including oesophageal carcinoma [16, 17, 29, 37].

P21$^{WAF1/CIP1}$ is a regulatory protein encoded by a gene on chromosome 6p. It interacts with a broad range of cyclin-cyclin dependent kinase (CDKs) complexes, binding to proliferating cell nuclear antigen and thus causing inhibition of DNA replication [7]. *P21* gene expression is directly up-regulated by wild-type *p53* gene at the transcriptional level and is considered to be a downstream effector of *p53*-induced G1 arrest [7]. In several tumour cell lines, it has been reported that the introduction of *p21* caused growth suppression and apoptosis [8], but the exact role of *p21* in cell proliferation or apoptosis of human malignant tumours remains undefined, especially in oesophageal carcinoma.

We have examined the expression of *p21* in resected tumours by immunohistochemical methods. We have analysed the relationship to alterations of *p53* gene, proliferative activity, frequency of apoptosis (apoptotic cell index) and the degree of differentiation of the carcinoma. Mutation of the *p53* gene was analysed by fluorescence-based single-strand conformation polymorphism (F-SSCP) analysis, proliferative activity was analysed by

Ki-67 immunostaining (Ki-67 labelling index), and the apoptotic cell index was analysed by in situ DNA nick end labelling.

Materials and methods

The present study was performed using 25 surgically resected specimens of oesophageal squamous cell carcinoma. Operations had been performed at Tokyo Medical and Dental University Hospital from 1994 to 1995. Clinical and pathological data are summarized in Table 1. Preoperative radiation or chemotherapy was not performed in any case. Fresh tumour tissue and normal epithelium were snap-frozen in liquid nitrogen and stored at –80°C until use for the immunohistochemical and molecular analyses. The degree of differentiation in each case was classified into three grades: well, moderately, and poorly differentiated types, according to the histological classification of the World Health Organization [39].

For immunohistochemistry, monoclonal antibodies against $p21^{WAF1/CIP1}$ (6B6, Pharmingen) and Ki-67 (MIB-1, Immunotech) were used. Monoclonal antibody against Ki-67 proved useful for both frozen and paraffin sections, showing almost the same immunoreactivity. However, monoclonal antibody against p21 was only useful for frozen sections. Immunoreactivity of p21 on paraffin sections was weak and unreproducible, even if microwave heating was performed as a pretreatment.

Frozen specimens were cut serially into sections 4 μm thick and were laid on poly-L-lysine-coated slides. Sections were fixed in acetone for 5 min, subsequently immersed in methanol containing 0.3% (v/v) H_2O_2 for 20 min, washed with PBS, incubated in normal rabbit serum for 10 min, and then reacted with primary antibody. 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. Sections incubated with normal mouse serum in place of the primary antibody were used as negative controls. Skin samples after radiation therapy were used as positive control for p21 and Ki-67 immunostaining.

In each case, the degree of p21 expression was calculated as the percentage of positive cells divided by the total number of examined cells of all examined fields. Faint or questionable reactions were regarded as negative in the present series. Fields were randomly selected from the area where carcinoma invasion was marked, and approximately 2,000 nuclei were counted in each case. Stromal cells positive for p21 were carefully excluded from the counting process. The Ki-67 labelling index (LI) was calculated in each case after counting approximately 2,000 nuclei in randomly selected fields. Counting of p21- or Ki-67-positive cells was performed independently by two experienced observers to avoid bias. Necrotic areas were not selected for the counting process.

In situ DNA nick end labelling was carried out by the method already described elsewhere [1]. Sections fixed in acetone were washed in Tris buffer. DNA 3′-end labelling with digoxygenin-ddUTP (dig-ddUTP) was performed at 37°C in a humidified chamber for 1 h. Antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-inodyl-phosphate; Boehringer Mannheim) for alkaline phosphatase were used for visualization of labelled cells. For positive controls, 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 after counting approximately 2,000 nuclei in randomly selected fields. To compare the distribution of positive cells, double staining for in situ DNA nick end labelling and immunostaining for p21 were also performed in a case of well-differentiated type.

Fluorescence-based single-strand conformation polymorphism (F-SSCP) analysis of the *p53* gene was carried out by a method based on the previously described one [23]. Frozen sections cut 20 μm thick were incubated at 50°C in extraction buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl, pH 9) containing 1 mg/ml proteinase K and 0.5% SDS overnight. After phenol and chloroform extractions, DNA was precipitated in ethanol and resuspended in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for storage. DNA from exons 5 through 8 of the *p53* gene was amplified using the polymerase chain reaction (PCR). Amplifications were performed in 10-μl volumes with 200 ng of genomic DNA and 1 μM primers, in PCR buffer (50 mM Tris, 3 mM $MgCl_2$, pH 9.0) con-

Table 1 Summary of the clinical data (*M* male, *F* female, *a* adventitia, *mp* muscularis propria, *LN meta* lymph node metastasis)

No. of case	Age	Sex	Depth of invasion	LNs meta	Differentiation
1	66	M	a	+	Good
2	71	F	a	+	Good
3	66	M	a	–	Good
4	69	M	a	+	Good
5	53	M	a	+	Good
6	72	F	a	+	Good
7	43	M	a	+	Good
8	48	F	a	+	Good
9	63	M	a	+	Moderate
10	67	M	mp	–	Moderate
11	69	M	a	+	Moderate
12	58	M	a	+	Moderate
13	58	M	a	–	Moderate
14	77	M	a	+	Moderate
15	55	M	a	+	Moderate
16	59	F	a	+	Moderate
17	55	M	a	+	Moderate
18	59	M	a	+	Poor
19	79	F	a	+	Poor
20	67	M	a	+	Poor
21	65	M	a	+	Poor
22	56	M	a	+	Poor
23	74	F	a	–	Poor
24	71	M	a	+	Poor
25	81	F	a	–	Poor

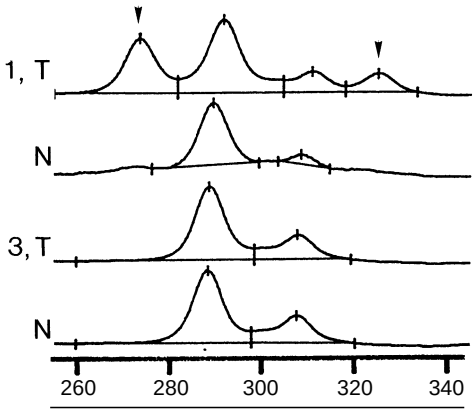


Fig. 1 Mutation of *p53* gene in oesophageal carcinoma. Exon 5 of the *p53* gene in carcinoma tissue (*T*) and normal tissue (*N*) was analysed by F-SSCP. The two peaks in normal tissue correspond to complementary strands of normal allele. *Arrowheads* in carcinoma tissue of case 1 indicate positions of strands of a mutated allele. Case 1 was judged positive and case 3, negative for mutation

taining 50 units of Taq polymerase (Perkin-Elmer/Cetus) for 35 cycles of 94°C denaturation (30 s), 55°C annealing (30 s), and 72°C extension (1 min) in an automated thermal cycler (Pharmacia). The oligonucleotide primers labelled at their 5′ ends with fluorescein-derivatives were purchased from Pharmacia. Primer sequences were:

exon 5 forward 5′-TTCCTCTTCCTGCAGTACTCC-3′
reverse 5′-AGCTGCTCACCATCGCTATCT-3′ (209 bp)
exon 6 forward 5′-TGATTCCTCACTGATTGCTCTTA-3′
reverse 5′-AGTTGCAAACCAGACCTCAGG-3′ (142 bp)
exon 7 forward 5′-GTGTTGCCTCCTAGGTTGGC-3′
reverse 5′-CAAGTGGCTCCTGACCTGGA-3′ (139 bp)
exon 8 forward 5′-CCTATCCTGAGTAGTGGTAATC-3′
reverse 5′-TTCTTGTCCTGCTTGCTTACCT-3′ (171 bp)

The PCR products (1 µl) were diluted in 79 µl of formamide dye solution, heated at 95°C for 5 min, and applied (5 µl/lane) to SSCP gel with glycerol fitted to an automated DNA sequencer (ALF, Pharmacia). During electrophoresis at 30 W the temperature of each gel was kept at 25°C with a built-in water jacket connected to an external thermostat-regulated water circulator. Fluorescent bands were quantitatively assessed with an automated sequencer, and cases showing abnormal extra peaks in comparison with normal epithelia were regarded as mutation positive (Fig. 1).

To evaluate the significance of difference in 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 p21 expression with status of *p53* gene, Fisher's exact test was used.

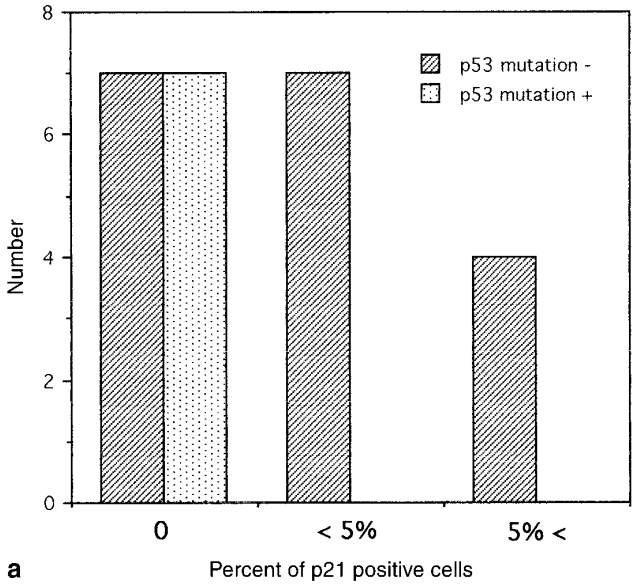
Results

Data from the molecular and immunohistochemical studies are summarized in Table 2. Of 25 cases, mutations of the *p53* gene were detected in 7 cases (28%) by F-SSCP analysis: mutation of exon 5, 3 cases; of exon 6, 2 cases; of exon 8, 2 cases. Expression of p21 was detected immunohistochemically in 11 of 25 cases (44%), the percentage of positive cells ranging between 1% and 10%. Of the 25 cases, 4 cases had over 5% of positive cells.

Figure 2a illustrates the relationship between p21 expression and status of the *p53* gene. All 7 *p53*-mutation-positive cases were negative for p21 expression. However, 11 of 18 mutation-negative cases (61%) were positive

Table 2 Summary of molecular, immunohistochemical and in situ DNA nick end labelling data

No. of case	*p53* Mutation (exon)	*p21* Expression (%)	Ki-67 labelling index (%)	Apoptotic cell index (%)
1	+ (5)	–	43.0	2.05
2	+ (8)	–	40.6	4.74
3	–	7.21	24.4	4.94
4	–	8.39	28.9	4.78
5	–	5.62	35.1	1.78
6	–	9.50	29.3	3.54
7	–	3.33	27.9	4.29
8	–	2.89	29.2	2.62
9	+ (5)	–	20.3	3.81
10	+ (8)	–	35.8	0.803
11	–	3.33	36.6	1.88
12	+ (6)	–	32.1	2.23
13	–	1.86	39.8	0.949
14	–	4.26	27.9	4.86
15	–	4.04	26.4	1.49
16	–	1.64	41.8	n.d.
17	–	–	35.2	1.32
18	+ (6)	–	34.1	0.954
19	+ (5)	–	29.5	1.27
20	–	–	27.9	1.33
21	–	–	34.8	0.845
22	–	–	19.1	n.d.
23	–	–	32.3	0.440
24	–	–	38.1	0.927
25	–	–	22.1	3.31



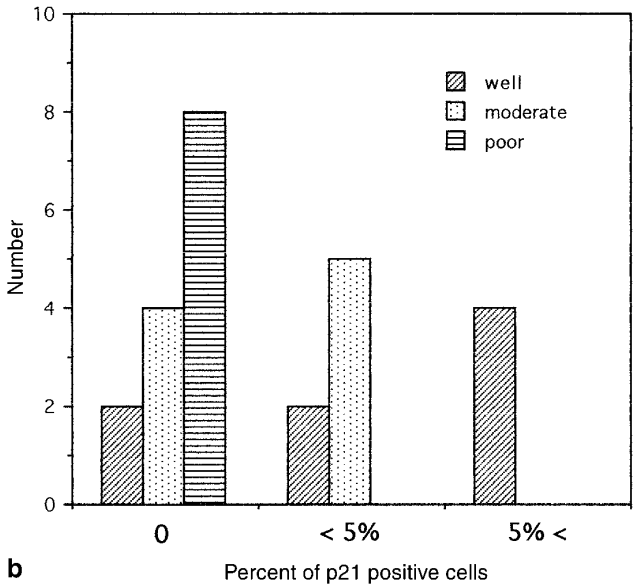


Fig. 2 Correlation of expression of *p21* with **a** *p53* mutations and **b** differentiation grade of cancer cells. **a** Relationship between *p21* expression and mutations of *p53* gene. All mutation-positive cases were negative for *p21* expression, whereas 11 out of 18 mutation-negative cases were positive for *p21* expression. **b** Relationship between *p21* expression and carcinoma differentiation. Well-differentiated type shows more intense expression than the poorly differentiated type, and moderately differentiated type shows an intermediate position between well and poorly differentiated types

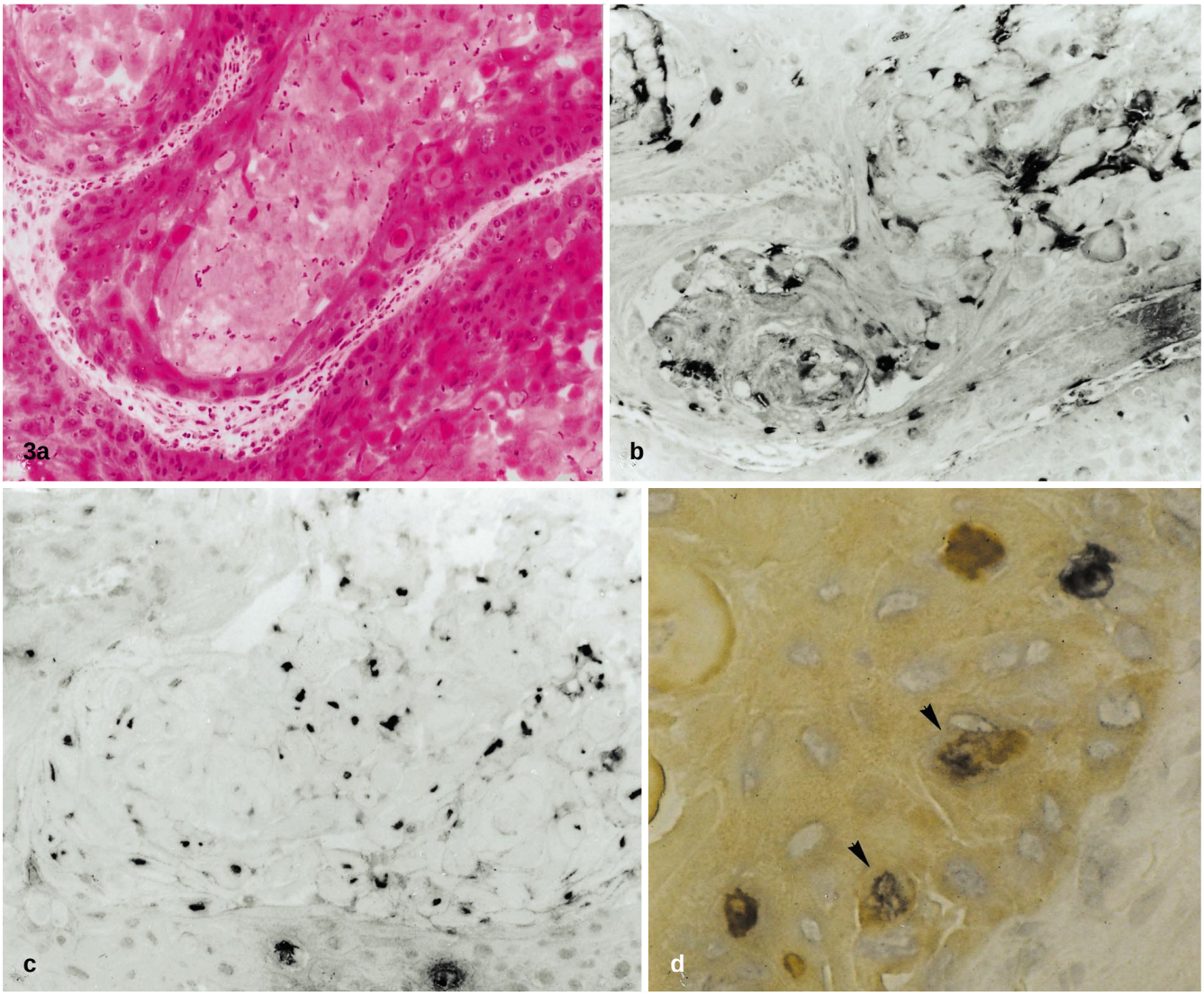


Fig. 3a–d Apoptosis and expression of *p21* in oesophageal carcinomas. **a** Well-differentiated oesophageal squamous cell carcinoma. Keratinization is marked in the centre of the invading nests. HE, ×33. **b** Immunostaining of *p21* in the same case as in **a**. Intranuclear reactivity is distributed in the middle layers of the invading nest, especially around the keratinization. ×33. **c** In situ DNA nick end labelling in the same case as in **a**. Apoptotic cells are also distributed in the middle layers, especially around the keratinization. ×33. **d** Double staining of in situ DNA nick end labelling and *p21* immunostaining. Nuclei of apoptotic cells were stained dark blue, and nuclei of *p21*-expressing cells were stained brown. In this focus, double-positive cells (*arrowheads*) are located in the middle layers of the nest, and single-positive cells are seen in the *right upper* and *left lower corners*. ×50

for p21 expression; the difference was statistically significant ($P<0.01$).

Figure 2b illustrates the relationship between p21 expression and degree of tumour differentiation. There was a good correlation between p21 expression and degree of tumour differentiation. Of 8 cases of well-differentiated type, p21 expression was detected in 6 cases (67%). Four cases had over 5% of positive cells. In contrast, all 8 cases of poorly differentiated type were negative for p21 expression. The 9 cases of moderately differentiated type demonstrated an intermediate position between well and poorly differentiated types: 5 of these cases were positive for p21 expression, the percentage of positive cells ranging between 1% and 5%.

Intranuclear expression of p21 was mainly scattered in the middle layers of the invading nests, especially around the keratinization (Fig. 3b). This distribution was quite different from that of Ki-67-positive cells, which, as proliferating cells, were mainly distributed in the peripheral fronts of the invading cell nests as previously reported [27]. Some p21-positive cells had pyknotic nuclei and others had swollen nuclei without pyknosis. The distribution of apoptotic cells was similar to that of p21-positive cells; they were distributed in the middle layers of the nest, especially around keratinization (Fig. 3c). However, simultaneous expression of p21 in the same apoptotic cells was seen in only a small portion of positive cells from the finding of double staining for p21 immunostaining and in situ DNA nick end labelling (Fig. 3d). In the normal oesophageal epithelium, intranu-

Table 3 Expression of *p21* and mutation of *p53* gene in relation to the median Ki-67 labelling index and the median apoptotic cell index

	Ki-67 labelling index (%)	Apoptotic cell index (%)
p21 Expression		
– (*n*=14)	33.2	1.32*
+ (*n*=11)	29.2	3.08 *
p53 Mutation		
– (*n*=18)	29.3	1.83
+ (*n*=7)	34.1	2.05

* $P<0.05$ (Mann-Whitney U-test)

clear expression of p21 was observed in all layers but the basal one. The intensity of the positive reaction was generally mild compared with the overt expression in carcinoma cells.

Data on the correlation of p21 expression with Ki-67 LI or apoptotic cell index are summarized in Table 3. Ki-67 LI was almost the same whether cases were positive or negative for p21 expression. However, the apoptotic cell index was significantly higher in the overt p21 expression group than in the no expression group (medians 3.08% vs 1.32%; $P<0.05$).

Correlations between the status of the *p53* gene and the Ki-67 LI or the apoptotic cell index are also summarized in Table 3. However, no statistically significant associations were observed with either index.

Discussion

The *p53* gene product is a well-known transcriptional factor implicated both in G1 cell cycle arrest after DNA damage and in apoptosis triggered under defined conditions [18, 19, 21]. P21 was discovered independently as a gene specifically activated by wild-type *p53* (WAF1) [7] and as an inhibitor of cyclin-dependent kinases (CIP1) [14]. The induction of p21 expression following radiation or chemical administration was reported to be dependent on wild-type *p53* and closely related to G1 arrest of the cell cycle [8]. These findings suggested that p21 may be a downstream effector of the growth-regulatory properties of the wild-type *p53*. In the upstream regulatory region of the *p21* gene, at least two highly conserved *p53*-binding sites exist, which might mediate transcriptional activation of *p21* [9]. However, it has been reported that p21 expression is also induced by a *p53*-independent pathway in other circumstances, as in the terminal differentiation of skeletal muscle cells and other cell lineages [13, 25, 30, 41]. In surgically resected samples of human malignant tumours, the relationship between the p21 expression and the status of the *p53* gene is still controversial. In pancreatic ductal carcinomas, it has been reported that p21 expression correlates neither with *p53* molecular status nor with *p53* protein expression [6]. Expression of p21 in pancreatic ductal carcinomas may be induced by a *p53*-independent pathway. Nevertheless, in soft tissue leiomyosarcomas all but one case with conserved *p53* function showed p21 expression and there was conserved p21 immunoreactivity in one case showing mutation of the *p53* gene [5]. Expression of p21 in leiomyosarcoma may be induced mainly by a *p53*-dependent pathway; the *p53*-independent pathway is minor. Normal oesophageal epithelium consistently showed mild expression of p21 in all but the basal layer, but in the carcinoma cells the degree of p21 expression varied from no expression to frequent and strong. Because molecular analyses have failed to demonstrate any abnormality of the *p21* gene in human tumours, the degree of p21 immunoreactivity is assumed to reflect the degree of expression of normal *p21* gene. Such overt expression of p21 was observed only in *p53*-mutation-negative cases, which indicates that p21 expression in oesophageal carcinoma is mainly induced by a *p53*-dependent pathway. There is still plenty of room for discussion of *p53*-independent induction, as a faint or questionable reaction for p21 was regarded as negative; only cases with overt expression were considered to be positive in this study, and the possibility of the homozygous deletion of the *p53* gene cannot be completely excluded. The present molecular analyses were performed on crude tumour extracts, and it may be that only unmutated *p53* gene derived from normal bystander cells might have been amplified in cases with homozygous deletion. The status of the *p53* gene in oesophageal squamous cell carcinomas has already been studied by SSCP and LOH analyses, but homozygous deletion has never been proved [17, 37]. Therefore, the possibility that homozygous deletion of the *p53* gene occurred in the present mutation-negative cases is considered to be very low.

The molecular mechanisms of apoptosis constitute an area of increasing interest, and recent studies have shown that at least two pathways mediate apoptosis. One is triggered by exposure of mouse thymocyte or other cell lineages to radiation or other agents associated with DNA damage [22] and is dependent on the induction of *p53*. The other pathway is independent of *p53* and follows exposure of mouse thymocytes to dexamethasone [3]. Several cellular proteins, such as bcl-2 [15], adenoviral E1B [38], and bcr-abl [10], have been identified as inhibitors of apoptosis. Other proteins, including bax [2, 28], adenoviral E1A [4, 31], and c-myc [11], have been identified as inducers of apoptosis. The interactions between these inducers and inhibitors are beginning to reveal a regulatory network. El-Deiry et al. demonstrated a close relationship between p21 upregulation and *p53*-dependent apoptosis in M3 variants of T-cell lymphoma and BAF3 immune haematopoietic cells [9]. Sheikh et al. reported that exogenously enforced overexpression of p21 induced giant cell formation and apoptosis in human breast carcinoma cell lines [32], and Shao et al. demonstrated that apoptosis in breast carcinoma cells by a novel retinoid was associated with *p53*-independent upregulation of p21 [33]. However, Steinman et al., in contrast, reported that p21 expression triggered by multiple differ-

entiation-inducing agents in haematopoietic cells through a *p53*-independent pathway was uncoupled from apoptosis and that upregulation of p21 mRNA did not occur during AraC-induced apoptosis [34]. Further, Zhang et al. reported that exogenous overexpression of p21 caused a significant decrease in the colony-forming ability of chronic myelogenous leukaemia cells without the morphological changes of apoptosis [41]. In the present study, apoptosis was assessed by the standard method of in situ DNA nick end labelling. Although a false-positive reaction due to double-strand break induced by DNA damage or necrosis and a false-negative due to extensive DNA clumping in apoptosis might have occurred in the assay, the apoptotic cell index was statistically higher in the overt p21 expression group than in the no expression group. Moreover, the distribution of apoptotic cells is also similar to that of p21-overexpressing cells, although the proportion of simultaneous expression of p21 in apoptotic cells was seen to be small on double staining. It is uncertain whether the result of double staining reflects a time lag between p21 expression and its execution or not, and apoptosis might be triggered by separate mechanism. However, the present data strongly suggest that endogenous p21 overexpression affects apoptosis in human oesophageal carcinoma cells.

The induction mechanisms of *p53*-dependent p21 overexpression are also an important subject, and Upadhyay et al. have reported that the *bcl-2* gene suppresses the expression of p21 in breast epithelial cells [36]. The relationship between p21 expression and the overexpression of *bcl-2* gene should be investigated in oesophageal carcinomas.

In the skeletal muscle cells and haematopoietic cells, a close association between p21 expression and terminal differentiation has been reported [13, 30]. El-Deiry et al. reported the topological distribution of p21-expressing cells in normal gastrointestinal epithelium and colonic neoplasms [9], and they reported a compartmentalization of p21-expressing cells in normal epithelium. They mean by this that the distribution of p21-expressing cells is separated from that of proliferating cells in normal epithelium. In colonic neoplasms, expression of p21 is decreased, and this distinct compartmentalization is disordered. In oesophageal squamous cell carcinoma, while the Ki-67 labelling index was found to be virtually identical in both p21-positive and p21-negative groups, p21 was absent in the proliferating cell fraction of the tumour. Expression of p21 might regulate the proliferation of tumour cells, and the preserved compartmentalization of p21-expressing cells might play an important part in the differentiation of squamous cell carcinomas. Further studies on the mechanisms of topological differences of p21 expression will lead to clarification of the relationship between tumour proliferation and differentiation.

In summary, *p53*-dependent induction of p21 appears to affect apoptosis and differentiation of carcinoma cells, as we have shown in surgically resected oesophageal carcinoma samples. Further studies of inducers of p21 overexpression and molecular pathways that connect p21 induction and apoptosis are needed to reveal the mechanisms of development and differentiation of oesophageal squamous cell carcinomas in detail.

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