Synergistic decline in expressions of p73 and p21 with invasion in esophageal cancers

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The significance of the *p73* gene, a homologue of the *p53* gene, in esophageal cancers is not fully understood. In order to clarify the role of p73 expression in esophageal cancers, p73 expression was immunohistochemically investigated in 106 surgically resected esophageal cancers and the results were compared with various clinicopathological factors. In normal esophageal epithelium, the expression of p73 was observed only in the nuclei of basal cells. In esophageal cancers, p73 immunoreactivity was observed in all intraepithelial lesions except one cancer, and was reduced with cancer invasion, to 78% and 64% at superficial invasion and deep invasion sites, respectively. However, p73 expression was not correlated with any other clinicopathological factor. The expressions of p53 and p21 were also investigated in esophageal cancer. To evaluate the status of the *p53* gene mutation immunohistochemically, two monoclonal antibodies (DO7 and PAb240) were used. There seemed to be an inverse correlation between p73 expression and p53 mutation. Moreover, the expression of p21 was highly correlated with p73 expression irrespective of the p53 mutation status. In human esophageal cancers, p73 expression decreased with increasing degree of tumor invasion, and its decreased expression in local advanced tumor caused down-regulation of p21 expression, which might reflect tumor progression. (Cancer Sci 2003; 94: 612–617)

Esophageal cancers have relatively high mortality rates, despite recent progress in cancer diagnosis and treatment.[1)] In Japan, long-term follow-up data of a total 11 642 esophagectomized cases show that the prognosis still remains poor, with a mean 5-year survival rate of approximately 36%, even if the esophagectomy is curative.[2)] Moreover, lymph node metastasis frequently occurs in esophageal cancers compared with other gastrointestinal malignancies, resulting in a poor outcome even for esophageal cancer patients detected at an early stage.[3,4)] About 40% of esophageal carcinomas with submucosal invasion have already metastasized to the lymph node,[3)] and only 60% of esophageal cancer patients with submucosal invasion survive for 5 years.[2)]

Recent advances in molecular biology have elucidated that various oncogenes and tumor suppressor genes are closely related to the development and progression of esophageal carcinomas.[5–11)] Inactivation of the INK4a locus or p16 is the most frequent genetic abnormality in esophageal cancers and occurs in the majority of esophageal cancers at the early stage of carcinogenesis.[12,13)] Loss or down-regulation of p16 expression in esophageal cancers is attributed to genetic changes such as homozygous deletion or point mutation in addition to epigenetic hypermethylation of the gene.[11)] Amplification of the *cyclin D1* gene is also a well-known genetic change in esophageal cancers and its overexpression is closely related with the invasiveness of cancer cells and the patient's outcome.[5,9)] Furthermore, *p53* gene mutation is detected in more than half of esophageal carcinomas and a large number of previous studies have revealed that p53 mutation plays an important role in cancer progression and its carcinogenesis,[14–17)] although the details remain unclear.

On the other hand, p73 has recently been identified as a structural and functional homologue of p53,[18)] and has been shown to have transcriptional activity for $p21^{WAF1}$ and to induce apoptosis *in vitro*.[19,20)] In early studies, an increased level of p73 expression was found in many human malignancies associated with p53 mutation.[21–25)] Therefore, it is thought that *p73* is unlikely to be a tumor suppressor gene. However, p73 expression is down-regulated in lymphoblastic lymphoma and Burkitt's lymphoma,[26,27)] neuroectodermal tumors[28)] and ovarian tumors,[29)] although somatic mutation of the *p73* gene is infrequent in various human cancers including esophageal carcinomas.[21,22)] Recently, the mechanism of down-regulation of p73 expression in these tumors has been ascribed to epigenetic hypermethylation on the promoter region of the gene.[26–28)]

Here, we clinicopathologically studied the role of p73 expression in esophageal cancers and tried to assess the compensatory relationship between p73 and p53 expressions through p21 expression.

Materials and Methods

Patients' characteristics and tissue preparation. Of 158 cases with primary esophageal carcinomas who underwent radical esophagectomy in the Department of Surgery 1, Gunma University Hospital, Japan, 106 were selected for the study; the others were excluded because of preoperative irradiation and/or chemotherapy. The tumor stage and disease grades were classified according to the 5th edition of the TNM classification of the International Union Against Cancer (UICC). The average age was 62.1±8.1 years, and the male to female sex ratio was 6:1. The localization of the main tumor in the esophagus was upper thoracic in 14 cases, middle thoracic in 64 cases and lower thoracic in 28 cases. The mean follow-up period for all cases was 32.3±34.9 months with a range of 2.7 to 192.2 months.

Immunohistochemistry. Surgically resected esophageal cancer tissue was fixed in 15% formalin and embedded in paraffin for routine pathological diagnosis. Paraffin blocks containing representative cancer tissue were selected and used for immunohistochemical study, and 3-μm-thick paraffin sections were serially cut and mounted on silane-coated glass slides. Immunohistochemical staining was performed according to the method described previously.[30)] Briefly, after deparaffinization in xylene and rehydration in a descending series of alcohol, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min, and antigen retrieval was carried out according to the method shown in Table 1. The sections were rinsed in phosphate-buffered saline (PBS, pH 7.4), and nonspecific antibody binding was blocked with 10% normal goat serum (for p73) or 10% normal horse serum (for the others) in PBS for 30 min at room temperature. Then, the sections were reacted with the primary antibodies (Table 1) for 24 h at 4°C,

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washed with PBS, and incubated with biotinylated secondary antibody for 30 min at room temperature, followed by treatment with the avidin-biotin peroxidase complex (DAKO, Glostrup, Denmark) for 30 min at room temperature. The sections were washed with PBS, and peroxidase activity was visualized with 0.03% 3,3′-diaminobenzidine tetrahydrochloride containing 0.06% H_2O_2 in 50 mmol/liter ammonium acetate-citric buffer (pH 6.0). Light nuclear counterstaining was carried out with Mayer's hematoxylin. Normal esophageal squamous epithelium was used as an immunohistochemically positive control for p73 and p21. Formalin-fixed and paraffin-embedded esophageal cancer cell lines T.T and T.Tn (T.T: JCRB 0262, T.Tn: JCRB 0261 purchased from Health Science Research Resources Bank, Tokyo) were also used as immunohistochemically positive controls for *p53* gene mutation.

Evaluation of immunohistochemical staining. Immunohistochemical results were separately evaluated by two pathologists (T.N. and K.K.). In each case, two cancerous areas, that is, the superficial invasion site and the deep invasion site, were evaluated for each protein expression. Another area of intraepithelial extension was also evaluated, if it was present in the tumor. Immunohistochemistry for p73 was evaluated as follows (Fig. 1): negative (−), when the nuclear staining for p73 was less than 5% of tumor cells; focally or peripherally positive (+), when p73-positive cells were 5−20% of tumor cells; diffusely positive (++), when p73-positive cells were more than 20% of tumor cells. For p21 staining, nuclear-positive cells were evaluated as the labeling index. The evaluation of p53 staining was as follows: positive (abnormal pattern), when more than 40% of tumor cells were positive for p53 by DO7 monoclonal antibody (MoAb) as well as by PAb240 MoAb; negative (normal), when less than 40% of tumor cells were positive for p53 by DO7 MoAb, and completely negative by PAb240 MoAb.

Statistical analysis. The relationship between the target protein expression and clinicopathological factors was analyzed using the χ^2 method, Fisher's exact test, and the ANOVA method. The relationship among p73, p53 and p21 expressions was determined with the Mann-Whitney test. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The significance of differences in survival was analyzed using the log-rank test in a univariate analysis.

Results

In the normal esophagus, p73 immunoreactivity was seen exclusively in the nuclei of basal cells of the squamous epithelium, while p21-positive nuclei were scattered in the parabasal area (Fig. 2, A, B). By p53 immunostaining using DO7 MoAb, faint and sporadic positive cells were seen in the nuclei of the basal cells of regenerated epithelium. However, no specific immunoreactivity with PAb240 MoAb was observed in the non-neoplastic esophageal epithelium (data not shown).

In esophageal cancers, p73 immunoreactivity had a tendency to be strong in mucosal carcinomas including early intraepithe-

Table 1. The primary antibodies used in this study

	Protein	Dilution	Methods of antigen retrieval
p73	Polyclonal antibody (CHEMICON)	1:2000	autoclaved in citrate buffer (pH 6.0), 121°C, 20 min
p21	Clone 4D10 (Novocastra)	1:50	microwaved in EDTA buffer (pH 8.0), 94°C, 20 min
p53	Clone DO7 (Novocastra)	1:100	microwaved in ZS buffer[1], 94°C, 20 min
	Clone PAb240 (NeoMarker)	1:50	boiled in EDTA buffer (pH 8.0), 99°C, 20 min

1) 20% Zinc sulfate heptahydrate in distilled water.

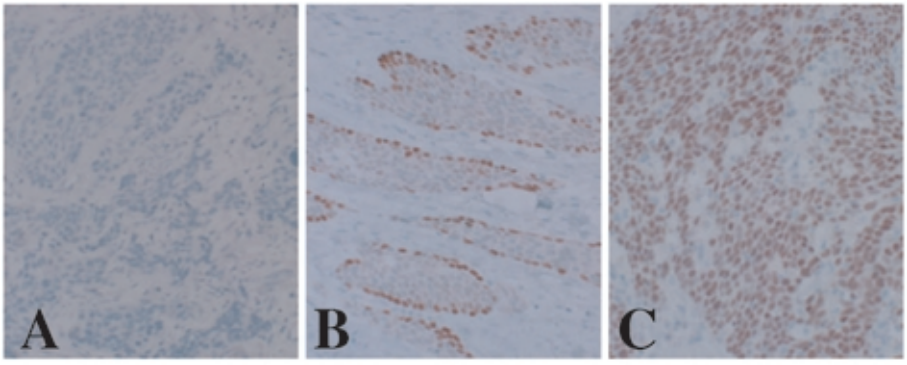

Fig. 1. Immunohistochemical evaluation of p73 expression. Negative (–) for p73 stain (A): no positively stained cells are observed. Focal/peripheral positive (+) (B): p73-positive nuclei are observed in the periphery of tumor nests. Diffuse positive (++) (C): the majority of cancer cells contained p73-positive nuclei.

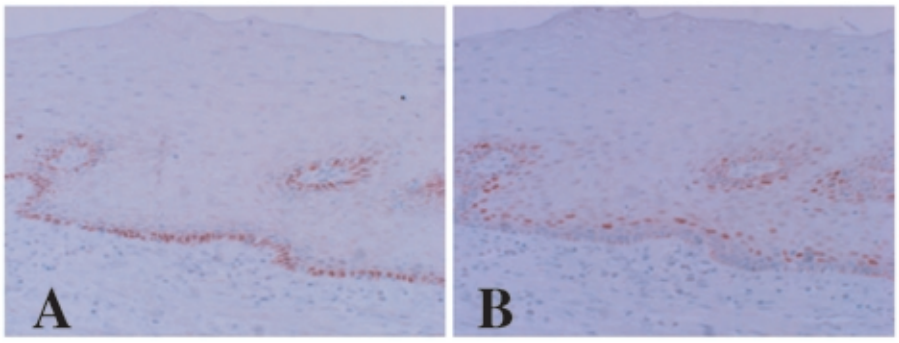

Fig. 2. Immunohistochemistry for p73 and p21 in normal esophageal epithelium. In the esophageal epithelium, p73 and p21 immunoreactivities were seen in the nuclei of basal cells (A) and suprabasal cells (B), respectively.

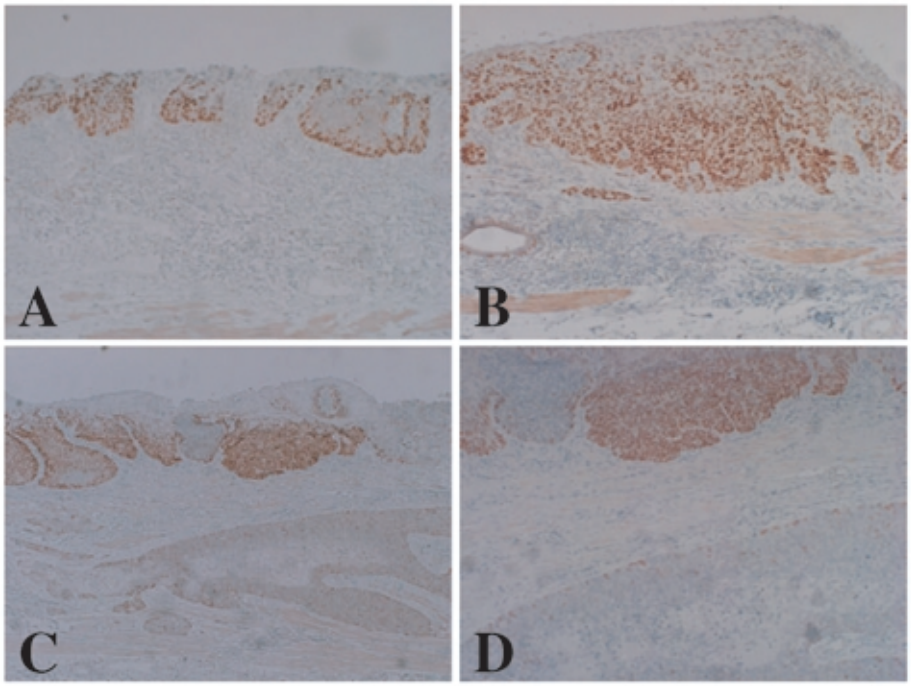

Fig. 3. p73 expression in various sites of cancer invasion. p73-positive nuclei were seen in the intraepithelial extension of esophageal cancers (A). Early invasive esophageal cancers showed strong p73 immunoreactivity (B). In esophageal cancers, strong p73 immunoreactivity was seen in cancer cells located in the superficial area (C, D) but not in the cancer nests located in deep invasive front (C, D).

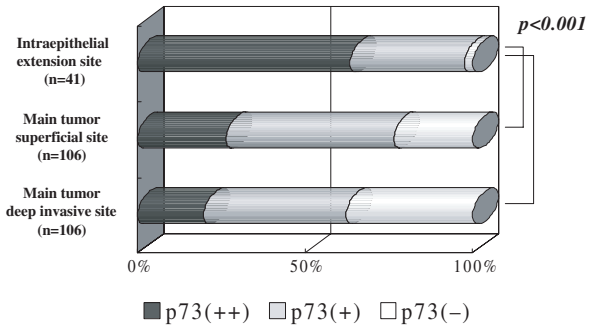

Fig. 4. The results of p73 expression in different invasion sites of esophageal cancers.

lial carcinomas. Of nine early intraepithelial carcinomas, seven showed diffusely positive staining for p73. However, p73-positive nuclei were gradually reduced in the invasive area of the tumor, especially in the deep invasion region (Fig. 3). As shown in Fig. 4, 41 of 106 esophageal carcinomas had intraepithelial cancer extension, and showed p73 immunoreactivity in all these lesions except for one case. Of p73-positive cases with an intraepithelial component, 26 (63%) were evaluated as diffusely positive (++) in these areas. On the contrary, p73 expression became negative (−) in 24% of the superficial invasion sites and in 38% of the deep invasion sites of the esophageal cancers investigated ($P<0.001$). In the superficial invasion sites, p73 immunoreactivity was preserved in 81 cases (76%), of which 53 and 28 cases were p73 (+) and p73 (++), respectively. In the deep invasion sites, 45 and 21 cases were p73 (+) and p73 (++), respectively. Careful examination of the immunohistochemical results showed that p73-positive nuclei were arranged along the periphery of the cancer nest in many cases (Fig. 1B). These cancer nests usually showed squamous differentiation toward keratinization and p21 immunoreactivity used to be localized inside the p73-positive cells. Moreover, cancer nests showing diffuse p73 and p21 staining rarely showed squamous differentiation. These staining patterns were frequently seen in intraepithelial cancers including mucosal cancers and intraepithelial extension of advanced cancers.

The immunohistochemical results for p73 in the deep invasion sites were compared with various clinicopathological factors, and the results are summarized in Table 2. There was no significant relationship between p73 immunoreactivity and age, gender, histology, depth of invasion, lymph node metastasis, pathological stage, vessel permeation, other pathological factors or the patient's outcome.

Using DO7 and PAb240 MoAbs, the expression of p53 was immunohistochemically investigated in 106 esophageal carcinomas. p53 detected by DO7 MoAb was diffusely stained in 54 carcinomas (52%), which were regarded as showing mutation of the *p53* gene. PAb240 MoAb positively stained 49 carcinomas, of which 45 (92%) were also diffusely positive for DO7 MoAb. Finally, p53 expression was evaluated as normal and abnormal in 61 and 45 esophageal cancers, respectively. Compared with the results of p73 immunohistochemistry, PAb240 MoAb positivity was inversely related to p73 immunoreactivity (Table 2). In addition to p53 staining, p21 immunohistochemistry was performed and evaluated as a labeling index in order to examine the influence of upstream p53 and p73 expressions. The mean labeling index of p21 staining was increased according to the degree of p73 expression (Table 2). Fig. 5 shows the relationship between p21 and p73 expressions on the basis of p53 PAb240 MoAb positivity in esophageal carcinomas. The p21 expression was significantly correlated with p73 expression, even in PAb240 MoAb-positive carcinomas

Discussion

In histologically normal squamous epithelium, p53 homologues including p73 show similar basal and/or parabasal expression, but that of p53 is weaker and discontinuous.[31] Moreover, p21, downstream from p53 homologues, is also expressed mainly in the parabasal cells of squamous epithelium.[32, 33] Our immunohistochemical study revealed that p73 and p21 were strongly positive in the nuclei of basal cells and suprabasal cells of normal esophageal epithelium, respectively. In the immunohistochemistry for p53, DO7 MoAb recognized both wild and

Table 2. Summary of clinicopathological factors of esophageal cancers used in this study

	p73 (–) *n*=40	p73 (+) *n*=45	p73 (++) *n*=21	Significance
Age	61.8±9.0	61.1±7.8	64.9±8.6	0.709
Gender				
Male/female	4/36	8/37	3/18	0.590
Histology[1)]				0.796
Well diff. SCC	9	9	7	
Mod diff. SCC	16	23	9	
Por diff. SCC	11	10	3	
Others[2)]	4	3	2	
Depth of invasion				0.402
pT0, 1	12	21	9	
pT2	10	4	2	
pT3	16	17	8	
pT4	2	3	2	
pN				
pN (–)/pN (+)	20/20	18/27	9/12	0.976
pStage				0.328
Stage 0, 1	10	14	7	
Stage 2	16	13	5	
Stage 3	6	12	5	
Stage 4	8	6	4	
Lymph vessel permeation				
ly (–)/ly (+)	13/27	15/30	7/14	0.996
Blood vessel permeation				
v (–)/v (+)	22/18	23/22	11/10	0.937
Growth pattern				
inf $\alpha/\beta/\gamma$	7/28/5	9/32/4	7/11/3	0.561
Intraepithelial extension				
ie (–)/ie (+)	18/22	24/21	13/8	0.440
Intramural extension				
im (–)/im (+)	35/5	38/7	19/2	0.482
p53 PAb240 staining				
PAb240 (–)/PAb240 (+)	28/12	22/23	7/14	*0.017*
p21 staining (%)	7.18±8.8	13.1±14.1	15.8±16.3	*0.009*

1) Well diff. SCC, well differentiated squamous cell carcinoma; mod diff. SCC, moderately differentiated squamous cell carcinoma; por diff. SCC, poorly differentiated squamous cell carcinoma.
2) Others: 2 small cell carcinoma cases, 2 adenosquamous carcinoma cases, 4 adenocarcinoma arising from Barrett esophagus cases, a basaloid squamous cell carcinoma case.

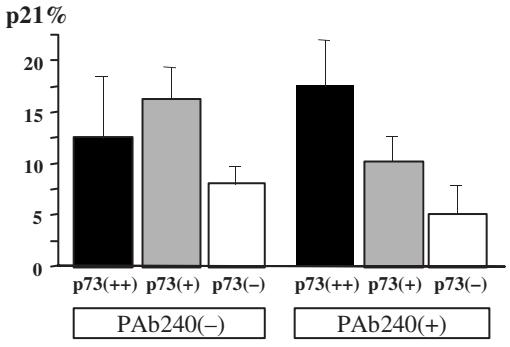

Fig. 5. The relationship between p21 and p73 expressions in esophageal cancers according to the p53 status.

mutant p53,[34–36)] but PAb240 MoAb only recognized mutant-type p53,[37)] so PAb240 MoAb failed to demonstrate any specific staining in the normal esophageal epithelium. In squamous epithelium, basal and parabasal proliferative compartments containing stem cells and transient amplifying cells have a crucial role in cell proliferation and differentiation.[38)] Recently, p63, one of the p53 homologues, has been shown to be a marker of stem cells of keratinocytes and to be expressed exclusively in the nuclei of basal cells.[39)] Expressions of p53, p73, p63 and p21 might be closely related to the clonal evolution or proliferative activity of esophageal squamous epithelium.

In esophageal carcinogenesis, cancer cells generally arise from a stem cell or transient amplifying cells that have suffered genetic and epigenetic alterations in oncogenes and tumor suppressor genes due to various carcinogenic stimuli.[38)] In early esophageal cancers, neoplastic cells proliferate in the basal and parabasal compartments of the epithelium, the early stage of which is called dysplasia or carcinoma *in situ* by pathologists.[40–42)] Our immunohistochemical study demonstrated that p73 and p21 expressions were conserved in superficially located cancer cells, including early intraepithelial and mucosal cancers and intraepithelial extension. Intraepithelial extension is defined as an intraepithelial carcinoma contiguous to invasive carcinoma, and it coexists in about 30% of squamous cell carcinomas of the esophagus.[43)] Intraepithelial extension had been considered a lateral intraepithelial spread from the main tumor on an earlier occasion.[44)] In fact, the incidences of intraepithelial extension gradually decrease according to tumor invasion, so that intraepithelial extension is surely an early lesion of the main tumor.[43)] The expression patterns of p73 and p21 in early esophageal cancer lesions might mimic those in normal basal and parabasal compartments of the squamous epi-

thelium irrespective of p53 mutation status. Morphologically, basal and parabasal cells do not usually show any differentiation to keratinocytes. If p73 and p21 stained diffusely in esophageal cancers, cancer cells usually showed no squamous differentiation, as seen in esophageal intraepithelial lesions. On the contrary, p21 expression instead of p73 expression might be associated with squamous differentiation or keratinization, similar to that in the clonal evolution of keratinocytes. A similar phenomenon has been reported in head and neck squamous epithelium and its cancers.[31]

In most human esophageal carcinomas, disruption of the p16-pRb pathway occurs early in their carcinogenesis, so that this phenomenon causes up-regulation of transcription factor E2F-1.[10, 12, 45] Recent studies have revealed that E2F-1 induces p73 as well as p14 at the transcriptional level.[46, 47] This might be one of the reasons why p73 was strongly expressed in the early lesions of human esophageal carcinomas in our immunohistochemical study. However, the expression of p73 was decreased with increasing degree of invasion, so inactivation of p73 may be attributed to cancer invasion or progression, as previously shown in head and neck cancers.[48, 49] Down-regulation of p73 expression decreases the induction of apoptosis and cell cycle arrest through down-regulation of p21 function,[19, 20] which may cause cancer cells to become more malignant.[50, 51] But, as shown in Table 2, p73 expression was not correlated with various clinicopathological factors relating to esophageal cancer progression or the patient's outcome. This is the first report on p73 expression in esophageal cancers and further studies will be necessary to reach a conclusion concerning the mechanism of down-regulation of p73 expression and the clinicopathological role of p73 expression in esophageal cancers.

p73 expression has been reported to compensate for the loss of function of p53 caused by mutation.[22–25] Therefore, we immunohistochemically evaluated p53 mutation in esophageal carcinomas using two MoAbs. Immunohistochemically, p53 mutation appeared as diffuse nuclear staining in more than 40% of cancer cells by immunohistochemistry using DO7 MoAb. PAb240 MoAb is known to react with only mutant-type p53 protein under non-denaturing conditions, but reacts with both mutant and wild-type p53 under denaturing conditions.[37] In esophageal cancers, evaluation of p53 mutation with the two MoAbs gave almost identical results. Comparing the p53 mutation with immunohistochemical p21 expression, no significant correlation was observed in the esophageal cancers investigated in this study. In esophageal squamous cell carcinoma, several reports have proposed that no correlation exists between the p53 mutation and loss of p21 expression.[50–52] Therefore, another route for the activation of $p21^{WAF1}$ independent of the p53 pathway has been suggested.[53] On the other hand, p73 expression has been found to be strongly correlated with p21 expression in recent studies.[18, 19] Our study clearly showed that p73 positivity is found more often in esophageal cancers with abnormal p53 expression than in those with normal p53 expression. Moreover, p21 expression was highly conserved in esophageal cancers with abnormal p73 expression as well as with p73 positivity. This is consistent with the hypothesis that p73 might have transcriptional activity for $p21^{WAF1}$ *in vitro*.[19, 20] These findings may explain why p73 acts as a compensatory mechanism for p53 tumor suppressor function in esophageal squamous cell carcinomas.

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