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Evidence that Expression of a Mutated *p53* Gene Attenuates Apoptotic Cell Death in Human Gastric Intestinal-type Carcinomas *in vivo*

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To examine *in vivo* the validity of the results of experiments *in vitro*, we analyzed the relationship between *p53* gene status and apoptotic cell death of human gastric intestinal-type adenocarcinomas. Surgical specimens were classified into two categories: 18 gastric cancers with nuclear p53 protein (A), and 17 gastric cancers without nuclear p53 protein (B). Polymerase chain reaction-single strand conformation polymorphism disclosed a shifted band that corresponded to a mutation in the *p53* gene in 13 cases (72%) in category A and 3 cases (18%) in category B, the frequency being significantly higher in the former ($P<0.05$). Apoptotic cells were identified from routinely stained sections and by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). The TUNEL index [TI: (the number of TUNEL-positive apoptotic cells/the total number of tumor cells) $\times 100$] was $3.8\pm1.4\%$ in category A and $4.9\pm1.2\%$ in category B, the value being significantly lower in the former ($P<0.05$). The proliferating cell nuclear antigen index, defined similarly to the TI, was $56.4\pm16.3\%$ in category A, and it was significantly higher than that in category B ($P<0.05$). The immunohistochemically detected expression of $p21^{CIP1/WAF1}$ did not differ between the two categories, while Bax-positive tumor cells were more frequently detected in category A. These results indicate that (1) expression of a mutated *p53* gene attenuates apoptotic cell death of gastric cancer, in accordance with the previous *in vitro* finding that *p53* gene mutation provides a possible selective advantage for tumor cell proliferation, and (2) apoptosis is related not only to expression of p53 and the stage of the cell cycle, but also to p53-independent and cell cycle-independent events.

Key words: Gastric carcinoma — Apoptosis — *p53* — TUNEL — PCR-SSCP

Alterations in and overexpression of the *p53* gene are common events in various human tumors. In gastric carcinomas, mutations in *p53* have been shown to occur at a high frequency, namely in 20 to 58% of the tumors examined.[1-5] Sano *et al.*[6] found a loss of heterozygosity at the p53 locus in 13 (68%) of 19 informative cases, regardless of the histological type of the gastric carcinoma. We discovered deletion of the *p53* gene in ten (77%) of 13 well-differentiated carcinomas by interphase cytogenetics using fluorescence *in situ* hybridization.[7] The precise role of the gene in gastric tumorigenesis and its biological significance are not well understood, although some authors have pointed out that mutations in *p53* are correlated with depth of invasion, stage, and poor clinical outcome.[8-12]

Recently, the p53 protein was shown to be required for induction of the apoptotic pathway triggered by oncogenic activation and the expression of cytotoxic genes.[13,14] The protein might sensitize damaged cells to apoptosis, acting to prevent the propagation of transforming mutations. $p21^{CIP1/WAF1}$, induced by the wild-type *p53* gene, might play a crucial role in this process.[15] Cells lacking a functional p53 protein would be refractory to this process of elimination and might proliferate more aggressively. The cited results were, however, obtained in primary cell cultures and transgenic mouse models. The role of the *p53* gene in tumorigenesis and progression of tumors also needs to be elucidated in surgical specimens of various human cancers. Attenuated apoptosis might imply that tumors have a selective advantage *in vivo* via a decrease in the incidence of cell death.[16] The relationship between *p53* gene status and apoptosis, however, has not been examined using surgical specimens, except for Wilms' tumors. Recently, Bardeesy *et al.*[17] examined seven Wilms' tumors and demonstrated that the number of apoptotic tumor cells was apparently smaller in some anaplastic variants carrying *p53* gene mutation than in non-anaplastic tumors carrying the wild-type *p53* gene. The progression of tumors needs to be analyzed on the basis of both proliferation (mitosis) and cell loss (apoptosis).

In this study, we examined the correlation between *p53* gene status and apoptosis in specimens of human gastric cancer. Our results support the hypothesis that disruption of cell turnover plays an important role in tumor progression and suggest that p53 might be involved in this process in gastric cancers.

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MATERIALS AND METHODS

We selected 35 gastric carcinomas from the files of our department by applying the following criteria: category A (18 cases), immunoreactivity specific for nuclear p53 protein in more than 50% of all the cancer cells; and category B (17 cases), no immunoreactivity specific for p53 protein in the cancer cells. Histologically, the samples consisted of one papillary, thirty-two tubular and two poorly differentiated adenocarcinomas, all corresponding to the intestinal type in Lauren's classification.[18] "Early" gastric cancer is defined as a carcinoma confined to the mucosa or to the mucosa and submucosa, regardless of the status of the lymph nodes.[19] "Advanced" gastric cancer is defined as a carcinoma with invasion beyond the submucosa. All specimens had been fixed in 10% formalin and embedded in paraffin wax.

Immunohistochemistry Dewaxed paraffin sections were immunostained by the streptavidin-biotin-peroxidase complex method with the following primary antibodies: monoclonal antibodies (mAbs) raised against p53 nuclear protein (BP53-12, diluted 1 : 50; Novocastra Laboratories Ltd., Newcastle, UK); mAbs raised against $p21^{CIP1/WAF1}$ protein [p21(187), diluted 1 : 1,000; Santa Cruz Biotechnology, Inc., Town, CA], mAbs against proliferating cell nuclear antigen (PCNA; PC10, diluted 1 : 200; MEDAC, Hamburg, Germany); and polyclonal antibodies against Bax protein [Bax(P-19); diluted 1 : 50; Santa Cruz Biotechnology, Inc.]. The microwave-based treatment for antigen retrieval was performed in all cases. Immunostaining was visualized with diaminobenzidine (DAB)-H_2O_2 solution and specimens were counterstained with methyl green or hematoxylin.

***In situ* nick end labeling** To detect DNA breaks *in situ*, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed by the method of Gavrieli *et al.*[20] After deparaffinization and rehydration, sections were incubated in a solution of 20 μg/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo) for 10–20 min at room temperature (RT). Endogenous peroxidase was inactivated by incubating sections in 2% H_2O_2 in methanol for 20 min at RT, followed by washing with distilled water. Then, terminal deoxynucleotidyl transferase (TdT) buffer solution (100 m*M* potassium cacodylate, 2 m*M* cobalt chloride, 0.2 m*M* dithiothreitol, pH 7.2) containing 0.3 U/μl TdT (Gibco BRL Life Technologies Inc., Gaithersburg, MD) and 0.04 nmol/μl biotinylated dUTP (Boehringer Mannheim/Yamanouchi) were added and the sections were covered with coverslips or "Parafilm" (American National Can, Greenwich, Chicago, IL), and then incubated in a humidified atmosphere for 60–120 min at 37°C. To stop the reaction, sections were incubated in TB buffer (300 m*M* sodium chloride, 30 m*M* sodium citrate) for 15 min at RT. They were subsequently incubated with peroxidase-labeled streptavidin for 30 min at RT, and finally stained with DAB-H_2O_2 solution. The sections were counterstained with methyl green.

To confirm the specificity of staining, positive controls were prepared by treatment with DNase I (Stratagene Co., La Jolla, CA) before the reaction with TdT. TUNEL signals were detected in nuclei of all cells, including stromal cells, that had been pretreated with DNase I for 10 min at RT. Negative controls were prepared by omission of TdT or the biotinylated substrate from the buffer solution, and negative results were obtained in all cases.

The frequency of apoptotic cells was determined as the TUNEL index (TI): the percentage of TUNEL-positive cells relative to the total number of tumor cells examined. Cells were examined at a magnification of 200× and more than 1,000 different tumor cells were analyzed for each specimen. The TUNEL-positive cells in the stroma and the epithelial lumen were not counted. The PCNA index (PI) was defined as the percentage of PCNA-positive cells relative to the total number of cells examined.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis PCR-SSCP analysis without a radiolabel was performed to examine point mutations in the *p53* gene. Genomic DNA was isolated from a few, 50-μm-thick, paraffin-embedded sections after trimming with a razor to avoid contamination by non-neoplastic cells as far as possible. The isolated tissue was digested with proteinase K (0.2 mg/ml) at 37°C, and then genomic DNA was extracted by the phenol/chloroform procedure. Primers for PCR were designed by reference to the nucleotide sequence of the *p53* gene from exon 5 to exon 8, which is considered to be a mutational hot spot. The primers and annealing temperatures are described elsewhere.[7] Optimally diluted DNA was subjected to 35–40 cycles of PCR. Products of PCR were confirmed by electrophoresis on 2% agarose or 15% polyacrylamide gels, followed by staining with ethidium bromide. The products of PCR (2 μl) were diluted in 5 μl of 95% formamide and heat-denatured at 95°C for 5 min. The products were loaded onto 20% and 15% polyacrylamide gels with or without 10% glycerol and were subjected to electrophoresis at 20°C for 4 h at 150 V. The gels were stained with 0.1% silver nitrate at 20°C for 20 min. Visualization of the silver-stained bands was achieved by reaction with 0.1% NaOH that contained 0.04% formaldehyde.

As positive and negative controls, DNA was extracted from the human cancer cell lines HSC-39 and KATO-III. The former has been shown to have a mutation in exon 7 of p53 and the latter has a deleted *p53* gene.[21,22]

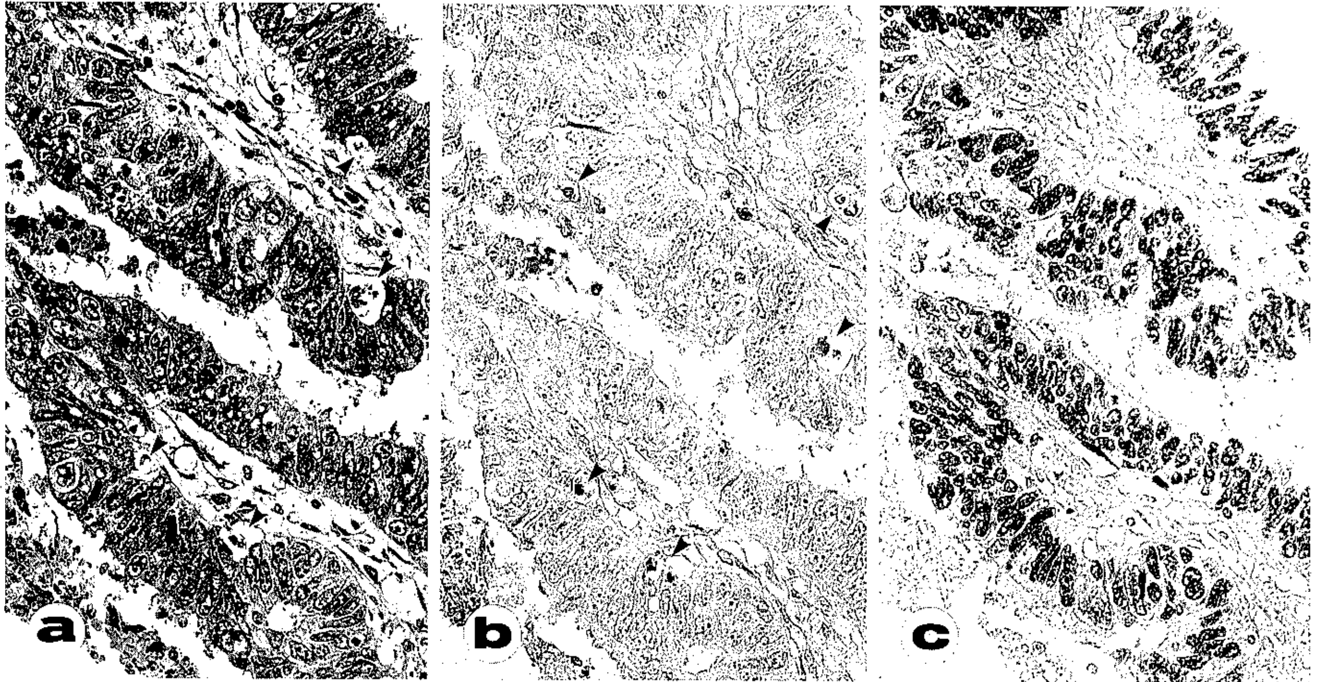

Fig. 1. Serial sections of p53-positive gastric tumors. a, A few apoptotic cells (arrowheads) are visible. HE staining. ×260. b, Apoptotic cells (arrowheads) with obvious positive TUNEL signals. TUNEL staining. ×260. c, Most cancer cells have been immunostained for the p53 protein in their nuclei. p53 immunostaining. ×260.

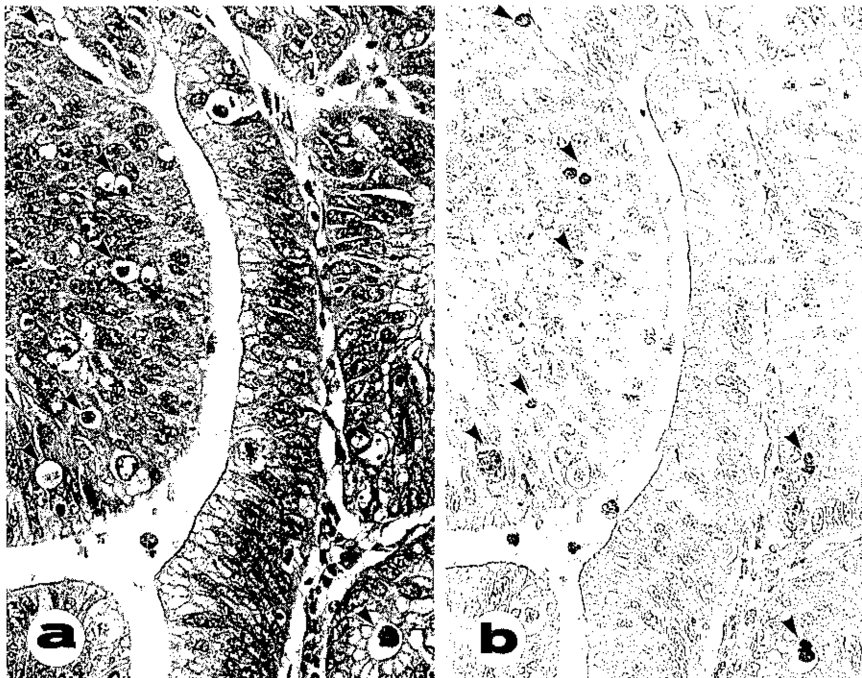

Fig. 2. Serial sections of p53-negative gastric tumors. a, Many apoptotic cells (arrowheads) are visible. HE staining. ×260. b, Apoptotic cells with TUNEL signals in their nuclei (arrowheads). TUNEL staining. ×260.

Table I. The Relationship between Expression of p53, Apoptotic Cell Death and Other Factors

	No. of cases	Exon that yielded abnormal band	Average TUNEL index (%)	Average PCNA index (%)
p53-positive cases	18		3.8±1.4	56.4±16.3
Early[a]	7	6, 7, 7, 8	3.9±1.6	44.6±17.1
Advanced[a]	11	5, 6, 7, 7, 7 7, 7, 8, 8	3.7±1.4	63.8±11.0
p53-negative cases	17		4.9±1.2	42.8±17.6
Early	10	5, 7	5.0±0.9	38.9±20.7
Advanced	7	5	4.8±1.5	48.4±10.9

* There is a significant difference (Student's *t* test; $P<0.05$).
TUNEL or PCNA index (%) = (number of positive cells)/(total number of tumor cells) × 100.
a) Early, early cancer with limited invasion of the mucosa or of the mucosa and submucosa. Advanced, advanced cancer with invasion beyond submucosa.

RESULTS

TUNEL-positive cells were observed most frequently in the basal portions of the cancer glands with variable distributions in both nuclear p53-positive (Fig. 1) and p53-negative carcinomas (Fig. 2). The TUNEL-positive cancer cells appeared on the serial hematoxylin and eosin sections as follows (Figs. 1a and 2a): 1) as a single structure with fragments of condensed chromatin, separated from the surrounding intact cells by a clear halo; and 2) as cells with a single nucleus that contained condensed chromatin and eosinophilic cytoplasm. Occasionally, normal-looking cancer cells with a non-pyknotic nucleus gave positive TUNEL signals. A few tumors contained apoptotic bodies known as nuclear fragments or dust with TUNEL signals in their lumen.

Table I summarizes the status of the *p53* gene, the TI and the PI for 18 nuclear p53-positive cases (category A), and 17 negative cases (category B). PCR-SSCP resulted in shifted bands in 13 cases (72%) in category A and in 3 cases (18%) in category B, and the frequency was significantly higher in the former ($P<0.05$). Shifted bands were detected that corresponded to mutation in exons 5 (one case), 6 (two cases), 7 (seven cases; Fig. 3) and 8 (three cases) in category A, and in exons 5 (two cases) and 7 (one case) in category B. Human gastric cancer cell lines were also analyzed as control materials. A shifted band was detected that corresponded to a mutation in exon 7 in HSC-39 cells, in contrast to the total absence of the gene in KATO-III cells (Fig. 3), as expected from previous reports.

The TI was 3.8±1.4% in category A and 4.9±1.2% in category B, the value being significantly lower in the former than the latter ($P<0.05$). There was no significant difference in TI between early and advanced carcinomas within both categories. PI was 56.4±16.3% in category A and 42.8±17.6% in category B, being significantly higher in the former than the latter ($P<0.05$). PI

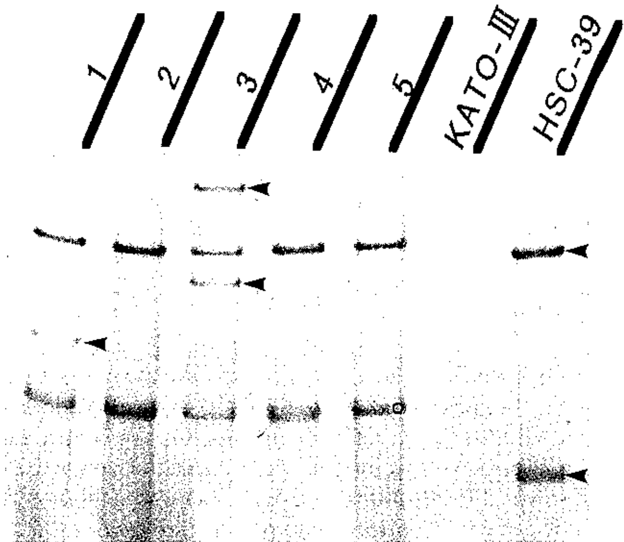

Fig. 3. PCR-SSCP analysis of point mutations in exon 7 of the *p53* gene. Shifted bands (arrowheads) are evident in cases 1 and 3 and in the case of HSC-39 cells. KATO-III cells yielded no detectable bands.

Table II. The Expression of p21 and Bax Protein in Human Gastric Carcinomas with and without Nuclear p53 Protein

p53 status	No. of cases showing immunoreactivity for p21				Bax			
	−	+	++	+++	−	+	++	+++
p53-positive (18 cases)	11	6	1	0	7	3	6	2
P53-negative (17 cases)	12	4	1	0	12	5	0	0

−, ≦5% positive cells, one or a few scattered positive cells without any clusters; +, 6–25% positive cells; ++, 26–50% positive cells; +++, ≧51% positive cells, usually strong and diffuse positive staining.

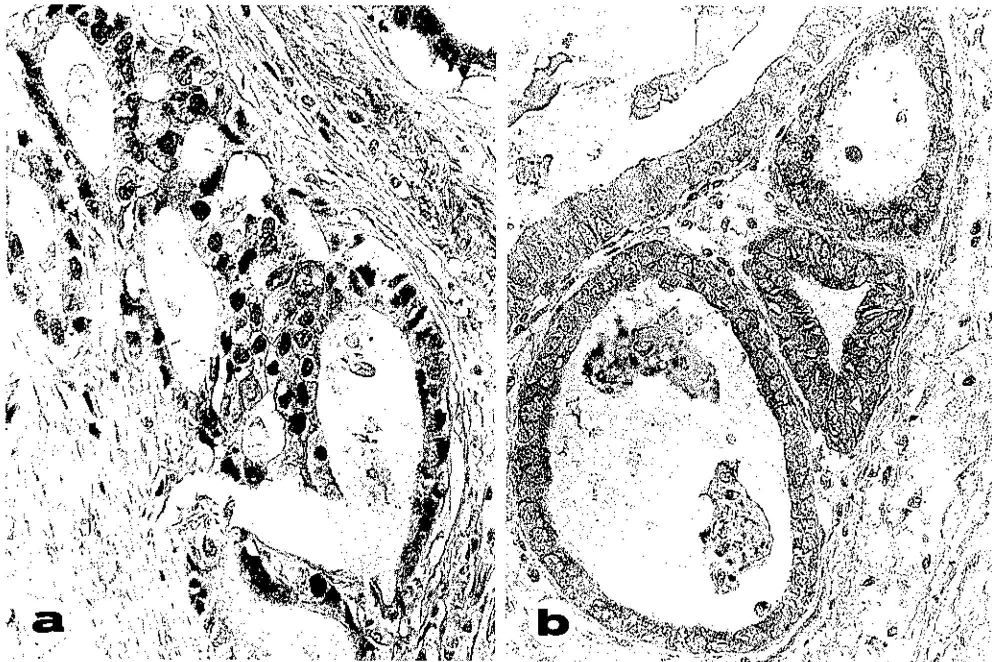

Fig. 4. p21 and Bax protein immunoreactivity in gastric cancer cells. a, Most cancer cells show strong immunoreactivity for p21 protein in their nuclei. p21 immunostaining. ×370. b, Bax protein is noted in the cytoplasm of most cancer cells. Bax immunostaining. ×370.

was significantly higher in advanced carcinomas than in early carcinomas in category A ($P<0.05$), while no significant difference was found in category B. Thus, nuclear p53-positive carcinomas were characterized as having a lower TI (an index of apoptosis) and a higher PI (an index of proliferation) than p53-negative carcinomas.

Table II shows the expression of $p21^{CIP1/WAF1}$ and Bax in categories A and B. Immunoreactivity specific for p21 was detected in the nuclei of cancer cells and that specific for Bax was found in the cytoplasm (Fig. 4). p21-positive tumor cells were found in 7 (39%) of the 18 carcinomas with nuclear p53 protein, and in 5 (29%) of 17 carcinomas without p53 protein. Bax-positive tumor cells were noted in 11 (61%) of the p53-positive carcinomas and in 5 (29%) of the p53-negative carcinomas, the frequency being significantly higher in the former than in the latter category ($P<0.05$).

DISCUSSION

The frequency of mutations in the *p53* gene was significantly higher in the gastric carcinomas in which nuclear p53 protein was found in most tumor cells than in those without the p53 protein. Our results are in good agreement with previous findings that a mutated *p53* gene is associated with expression of the gene product in a variety of human carcinomas.[17, 23-26] The mutated p53 protein has been demonstrated to accumulate in the nucleus via binding to oncogenic proteins or as a result of a prolonged half-life.[27, 28] The five cases of expression of the p53 protein without a shifted band suggest that some false-negative results might have been obtained in the analysis by PCR-SSCP. A non-shifted band does not necessarily imply the existence of a wild-type *p53* gene, as many authors have found.[29-31] By contrast, in three cases, no expression of p53 protein was detected, in spite of a shifted band of DNA. This result might reflect the existence of a silent mutation, a nonsense mutation or heterogeneity of the gastric cancer, with colonies of cells with and without a mutation in the *p53* gene.[7]

Apoptosis can occur in a cell cycle-dependent or cell cycle-independent manner.[32, 33] In the former case, apoptosis is induced in cells late in the G1 or in the G2 phase. Recent studies have demonstrated that the stability of wild-type p53 protein increases transiently in response to DNA damage by irradiation, and that the stabilized protein mediates arrest of the cell cycle in G1. Moreover, overexpression of wild-type p53 has been shown to sensitize transformed cells to apoptosis.[34] Conversely, cells with damaged DNA cannot undergo apoptosis when the *p53* gene is inactivated.[35] However, defects in the *p53* gene are associated with attenuated apoptosis and

chemo- and radioresistance in a mouse sarcoma model.[36] Our results support those observations. Mean apoptotic indices were significantly lower in gastric cancers that expressed the nuclear p53 oncoprotein than in those that did not express it. As mentioned above, the former might be considered to express a mutated p53 protein that fails to induce G1 arrest, with resultant enhanced survival, which could give the DNA-damaged cancer cells a selective advantage. Similar results were obtained by Kobayashi *et al.*[37] who examined the correlation between expression of p53 and apoptosis in human colonic adenomas and carcinomas. They found that the incidence of apoptosis was significantly lower in colonic tumors with widely distributed expression of p53 than in those with sporadic expression of the protein.

We cannot ignore the mean apoptotic index of the tumors in category B that did not express nuclear p53 protein. Although the difference in the apoptotic indices of the two categories was statistically significant, it was not as large as we had initially expected. Thus, apoptotic cell death might occur via expression of the *p53* gene or in a cell cycle-dependent or -independent manner in gastric tumors *in vivo*. It seems that apoptosis is not induced solely by overexpression of wild-type *p53*; the accumulation of additional oncogenetic insults is probably required to actuate the process of cell death.

Few comparative studies of proliferation and apoptosis in human gastric carcinomas have been reported. In the present study, the PI was significantly higher in advanced carcinomas than in early carcinomas in category A with nuclear p53 protein, implying clonal expansion with progression of the tumor. Thus, human gastric cancers with a mutation in the *p53* gene might be characterized by a lower incidence of apoptosis and higher proliferative activity, reflecting a possibly aggressive nature. Indeed, clinical observations indicate that mutations in *p53* are correlated with depth of invasion, stage, and poor clinical outcome.[11, 12]

The growth-inhibitory protein p21$^{CIP1/WAF1}$ is a potent inhibitor of various cyclin-dependent kinases, the expression of which is regulated at the transcriptional level in a p53-dependent and -independent manner.[38] The *p21* gene is induced by DNA-damaging agents that trigger G1 arrest in cells with wild-type p53, but not in cells with mutated p53.[39] A higher frequency of p21$^{CIP1/WAF1}$-positive cells might be expected, therefore, in p53-negative, non-mutated tumors. In the present study, however, the frequency of cases with p21 protein-positive tumor cells was rather higher in carcinomas with expression of p53 protein, implying a mutation of the gene. This result suggests that expression of p21 might be triggered by a p53-independent pathway in human gastric carcinoma, although the absence of immunoreactivity does not necessarily imply the total absence of the antigen, especially in paraffin-embedded sections. In fact, recent studies indicate the existence of a p53-independent pathway for induction of the expression of p21.[40–42] Similarly, the *Bax* gene has been shown to be activated by the product of the *p53* gene. The Bax protein promotes apoptosis, in contrast to Bcl-2, which prevents apoptosis.[43] Paradoxically, Bax-positive tumor cells were frequently detected in the carcinomas with nuclear p53, which were less frequently apoptotic. This phenomenon might reflect the diverse mechanisms of induction of apoptosis, with some not involving the *p53* gene.

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