Down-expression of tumor protein p53-induced nuclear protein 1 in human gastric cancer

The Down-Expression of Tumor Protein 53-induced Nuclear Protein 1 (TP53INP1) in Human Gastric Cancer

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Running title: TP53INP1 in gastric cancer

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ABSTRACT

Purpose: Overexpression of TP53INP1 induces G1 cell cycle arrest and increases *p53*-mediated apoptosis. To clarify the clinical importance of TP53INP1, we analyzed TP53INP1 and p53 expression in gastric cancer.

Experimental Design: TP53INP1 and p53 expression were examined using immunohistochemistry on 142 cases of gastric cancer. The apoptosis of gastric cancer cells was analyzed using the TUNEL method.

Results: TP53INP1 was expressed in 98% (139/142 cases) of non-cancerous gastric tissues and was down-expressed in 64% (91/142 cases) of gastric cancer lesions from the same patients. TP53INP1 expression was significantly decreased (43.9%) in poorly differentiated adenocarcinoma compared with well or moderately differentiated adenocarcinoma (81.6%). Cancers invading the submucosa or deeper showed lower positively (59.1%) compared with mucosal cancers (85.2%). Decrease or loss of TP53INP1 expression was significantly correlated with lymphatic invasion (54.3% vs 82.0% without lymphatic invasion) and node-positive patients (31.3% vs 68.3% in node-negative patients). P53 was expressed in 68 (47.9%) patients of gastric cancer, whereas it is absent in normal gastric tissues. A significant association was also observed between TP53INP1 status and the level of apoptosis in tumoral cells: the apoptotic index in TP53INP1-positive tissues was significantly higher than that in TP53INP1-negative portions. Finally, when survival data were analyzed, loss of TP53INP1 expression had a significant effect on predicting a poor prognosis (p=0.0006).

Conclusions: TP53INP1-positive rate decreased with the progression of gastric cancer. TP53INP1 protein negativity was significantly associated with aggressive pathological phenotypes of gastric cancer. TP53INP1 was related to the apoptosis of gastric cancer cells. The decreased expression of the TP53INP1 protein may reflect the malignant grade of gastric cancer and is regarded as an adverse prognostic factor.

Key words: **tumor protein 53-induced nuclear protein 1, p53, gastric cancer.**

INTRODUCTION

Tumor Protein 53-Induced Nuclear Protein 1 (*TP53INP1*) is a p53-inducible gene encoding two protein isoforms able to modulate p53 biological activities (1-4). TP53INP1 expression is strongly induced *in vivo* in mice with acute pancreatitis (1), and *in vitro* in several cell lines submitted to various stress agents (2，4). Over-expression of TP53INP1 induces cell cycle arrest in G1 phase and enhances the p53-mediated apoptosis (3). TP53INP1 co-localizes with p53 and the serine-threonine p53-kinase HIPK2 (5) within the promyelocytic leukemia protein nuclear bodies (PML-NBs) and physically interacts with these proteins modifying the p53 transcriptional activity on several p53 target genes (3). *TP53INP1* thus appears as a key-element in p53-mediated cell death and cell cycle arrest, induced by cellular stresses.

The multistep model of carcinogenesis in gastric cancer, the second most common cancer leading death in the world, suggests accumulation of genetic alterations, epigenetic changes and posttranslational modifications. It often metastasizes to other organs, including liver, lung, and ovary (6). Multiple factors are known to be related to gastric carcinogenesis, including Epstein-Barr virus (7) and Helicobacter pylori infections (8), microsatellite instability (9). From the molecular point of view, it has now been established that gastric carcinogenesis involved accumulation of mutations in oncogenes and tumor suppressor genes controlling epithelial cell growth and differentiation (10-14). In particular, TP53 mutations are frequently seen in gastric cancers and correlates with gastric cancer prognosis (15, 16). However, the molecular alterations and their role in gastric cancer still remain to be fully defined.

Previous works implied that *TP53INP1* is a proapoptotic gene induced by p53 (2). Overexpression of TP53INP1 promotes G1 arrest and apoptosis through the p53-mediated pathway (3). The aim of the present study was to analyze the expression patterns of TP53INP1 in a large series of gastric

carcinomas to (1) identify the possible modulation of TP53INP1 expression; (2) investigate the association with apoptotic activity; (3) analyze the relationship with clinicopathologic parameters, and evaluate its prognostic value.

MATERIALS AND METHODS

Patients and Specimens. One hundred and forty-two patients with gastric cancer were enrolled in this study. The areas adjacent to cancer lesions were used as non-malignant gastric tissue. The patients underwent operation at the Cancer Research Institute Hospital, Kanazawa University. The histological classification was defined using the Japanese classification of gastric carcinoma (17). Intestinal type was defined as either papillary or well to moderately differentiated tubular adenocarcinoma. Diffuse type was defined as poorly differentiated adenocarcinoma, signet-ring cell carcinoma, or mucinous adenocarcinoma. The series included 104 men and 38 women, and the mean age was 63.1±10.6 years. There were 76 and 66 cases of differentiated and undifferentiated type, respectively.

Immunohistochemistry. A standard avidin-biotin-peroxidase complex method (ABC) was used for immunostaining. Deparaffinized sections were treated by microwaving at a high power for 5 min two times in a 10 mM citrate buffer to retrieve antigenicity. After washing with PBS, the sections were immersed in 3% hydrogen peroxide in methanol for 20 min to block any endogenous peroxides activity. Then the ABC staining system kit (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used for detection. Sections were incubated with 10% normal serum for 1 hour to inhibit nonspecific antibody binding. Then, sections were incubated overnight at 4ºC with 6μg/ml of rat anti-human monoclonal antibody raised against to *TP53INP1* (kindly provided by A. Carrier). After washing with PBS, detection was done by successively incubating the sections with biotinylated goat anti-rat IgG for 30 min, and avidin-biotin-HRP for 30 min. After extensive washings with PBS, sections were stained with 3-diaminobenzidine for 2~10 min. Then, sections were counterstained with hematoxylin, dehydrated, and mounted. Nuclei were lightly counterstained with Mayer's hematoxylin. TP53INP1-positive cells were counted in fields chosen at random (100× magnification), and the percentage of the number of positive cells per 1,000 cells was expressed

as TP53INP1-positive index (%). Use the same method we counted the TP53INP-positive in nucleus and in cytoplasm under the microscopy with a 200 magnifying. The Normal IgG was used as a negative control.

P53 was immunolocalized using a DAKO LSAB kit (DakoCytomation, Japan, Kyoto, Japan). The primary antibody was rabbit monoclonal antibody against human p53 (Nichirei Inc, Tokyo, Japan). The procedure was according to the protocol from company. Final t he sections were incubated with DAB substrate as a chromogen. The cell nuclei were also lightly counterstained with Mayer hematoxylin.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). TUNEL-positive epithelial cells were detected on the sections using ApopTag Plus peroxides in situ apoptosis detection kit (Chemicon International, Inc., Temecula, CA, USA). Briefly, after pretreatment with 20 μg/ml of proteinase K and 3% hydrogen peroxide, sections were incubated with a labeling mixture for 1 hour at 37ºC. Then 55 μl of anti-digoxigenin-peroxidase were deposited on sections and incubated for 30 minutes. The reaction products were visualized by 3,3-diaminobenzidine substrate. Nuclei were counterstained with methy1 green for 10 minutes. After washing with n-butanol, the sections were dehydrated, and mounted. Apoptotic index (%) corresponding to the number of labeled nuclei per 1,000 nuclei was calculated.

Statistical Analyses. Experimental results were expressed as mean ± SEM. Difference between the means was evaluated by the Mann-Whitney U test. $P < 0.05$ was considered as statistically significant. The statistical analysis in this paper such as Kaplan-Meier analysis and Cox regression model was performed by using the software of StatView-5.0 Macintosh （Tokyo, Japan）.

RESULTS

TP53INP1 was expressed in non-malignant gastric tissues and its expression was reduced in gastric cancer tissues. In the non-neoplastic gastric mucosa, TP53INP1 was mainly located in the cytoplasm of epithelial cells (Fig. 1A and 1B). Some nuclei were also stained for TP53INP1 (Fig. 2). Similar patterns were observed for intestinal metaplasia samples (Fig. 1A, insert). To determine if TP53INP1 is differentially expressed in gastric carcinomas or if it is stage-related, we did immunohistochemical analysis on 142 samples (76 cases of intestinal type, and 66 cases of diffuse type). All the cancer samples had accompanying non-malignant tissues, 98% of them were positive with TP53INP1 expression (Table 1) and thus could be used as internal control. In contrast, the expression of TP53INP1 protein was seen in 91 cases (64%) showed TP53INP1 staining, the other 51 samples (36%) were found TP53INP1-negative (Fig. 1B, arrow). Overall, TP53INP1 expression in gastric cancers was significantly lower both in cell cytoplasm and nucleus than in non-malignant gastric tissue ($p<0.0001$, Table1; Fig. 2). However, the expression of TP53INP1 was decreased in well-differentiated tubular adenocarcinoma (Fig. 1C), and was markedly diminished in poorly differentiated-type cancer (Fig. 1D).

We next examined whether TP53INP1 expression is associated or not with development and progression of gastric carcinoma. The clinical details of the cohort of patients and the statistical analysis are listed in Table 2. Only two non significant associations were observed, i.e., age and gender. TP53INP1 negativity was associated with gastric body and antrum tumor location ($p=0.0193$), with poorly differentiated adenocarcinoma (diffuse type) ($p<0.0001$). With regard to the depth of invasion, the positive TP53INP1 expression rate was 100% intramucosal tumors (5/5), 81.8% when mucosa was invaded (18/22), 76.5% in muscularis propria (26/34), 54.3% in subserosa (25/46), and 48.6% in serosa (17/35). These results clearly show that alteration of TP53INP1 expression

was correlated to the staging of the tumors. The difference was statistically significant when T1 tumors were compared to the other stages (p=0.0111, Table 2). In addition, TP53INP1 was significantly expressed in node-negative patients (p=0.0037), and significantly associated with lymphatic invasion-negative patients (p=0.0010). Taken together, these results indicated that loss of TP53INP1 expression was significantly associated with poorly differentiated histology, deep invasion, lymph node invasion, and metastasis.

TP53INP1 and apoptosis. *TP53INP1* modulates the cell cycle arrest and programmed cell death (3). To investigate whether the modulation of TP53INP1 expression is associated with differences in apoptotic activity, TUNEL assays were done in all the 142 cases. TUNEL-positive nuclei were clearly seen in TP53INP1- positive (Fig. 3A) and negative (Fig. 3B) cancer lesions. As shown in Fig. 3C, the apoptotic index in the TP53INP1-positive group (7.48%±2.66%) was significantly higher than that found in the TP53INP1-negative group (4.16% ±2.41%).

TP53INP1 expression and prognosis. On univariate analysis, patient survival according to pathological stage was significantly different between TP53INP1- positive and TP53INP1- negative groups. Those patients with TP53INP1- positive expression had significantly better survival than those without TP53INP1 expression (p=0.0006, Fig. 4A). Survival for TP53INP1- positive patients with poorly differentiated adenocarcinoma was significantly longer than that of TP53INP1- negative patients (p=0.0199, Fig. 4B), whereas the survival of TP53INP1- positive patients in well or moderately differentiated adenocarcinoma was not significantly different from that of TP53INP1- negative patients (p=0.1110, Fig. 4C). Taken together, the results indicate that alteration of TP53INP1 expression was associated with a poor prognosis.

Nevertheless, no prognostic value for TP53INP1 expression was evidenced from the multivariate analysis (Table 3). Histological type, apoptotic index, metastasis, and lymph node invasion were the most important independent prognostic factors, TP53INP1 could not be considered as an independent prognostic marker.

P53 was not expressed in non-malignant gastric tissues and was expressed in gastric cancer tissues. AS show on Table 1 and figure 1E, the p53 protein was expressed in gastric cancer regions whereas it's absent in non-malignant portions. The staining was nuclear. Cytoplasmic staining without nuclear staining was regarded as negative. The p53 positive rate in gastric cancer was 47.9% that is significantly higher than that in non-malignant tissues.

DISCUSSION

The development and progression of gastric cancer involves many types of genes that need to be activated or inactivated in order to promote malignancy. Gastric cancer is a heterogeneous pathology, classified into 2 general subtypes: intestinal (differentiated) and diffuse (undifferentiated) (17). The intestinal type gastric carcinoma presents tumor suppressor gene alterations similar to colorectal tumors and distinct from diffuse type gastric cancer (18). An accumulation of multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules, and growth factor/receptor systems are involved during the multistep conversion from normal epithelial cells to clinical gastric cancer (10-14). *TP53* gene alterations have been observed in both histological subtypes (19). TP53INP1 is a tumor suppressor gene, located on the chromosome band 8q22 (20). Its expression is dependent on the activation of wide-type p53 (3).

In this study, we showed that TP53INP1 protein expression was significantly reduced in gastric cancer cells compared with non-cancerous adjacent tissues. We also reported that reduced TP53INP1 expression was associated with the diffuse cancer phenotype. Tomasini et al. (2) have shown that TP53INP1 and HIPK2 are partners in regulating p53 activity. It is increasingly evident that methylation of CpG islands in the promoter of specific tumor suppressor genes, such as *p16*, is associated with their silencing in human gastric cancer (21). The 5′ -upstream region of TP53INP1 contains a CpG island. The sequences from nucleotide-792, the region on exon 1 and part of the first intron, to nucleotide +839, has the highest content of CpG dinucleotides. However, there is no mutation of TP53INP1 gene in pancreatic carcinoma (unpublished data). In addition, the reduced TP53INP1 expression in gastric cancer especially in the diffuse type may relate to the wide type p53 inactivation in gastric cancer.

We showed p53 was expressed in 68 cases of gastric cancer, whereas it was not present in normal gastric epithelial. We observed that in non-gastric cancer regions the expression

of TP53INP1 was opposite to that of p53. The expression of TP53INP1 was dependent on the wild type p53. Since the wild-type p53 protein is biologically unstable and has a shorter half-life than mutant p53 protein (24). This characteristic of wide-type p53 protein does not allow it detected by immunohistochemical, but mutant p53 can be detected by immunostaining. Our data was similar to Carvalho et al. (22) showed that there is no different of the p53 expression between the intestinal type and the diffuse type in gastric cancer. Whereas Lin et al (23) showed the expression of p53 in the intestinal type is more frequent than that in diffuse type. The precise mechanisms of TP53INP1 suppression in gastric cancer need further research.

The mechanism of the suppression of these genes in poorly differentiated gastric carcinoma is not clear. Many studies suggested that most tumor suppressor genes play a role in mediating cell cycle arrest in the G_1 phase following DNA damage and also function in the removal of damaged cells by initiating apoptosis in certain physiological situations (10). TP53INP1 and HIPK2 are partners in regulating p53 activity (2). Overexpression of TP53INP1 induces G1 cell cycle arrest. TP53INP1 expression was significantly decreased in advanced gastric cancers. These results suggest that decrease of TP53INP1 expression might play an important role in the progression of gastric cancer. Assessment of TP53INP1 expression level may serve as a novel biomarker for predicting the malignant grade of cancers, like another marker for poor prognosis genes (25, 26).

Lymphatic involvement is thought to be important as an initial step of lymph node metastasis (27). Our study showed that TP53INP1 expression was significantly reduced in lymphatic invasion-positive groups. TP53INP1 expression decreased in node-positive. Taken together, these results suggested that loss of TP53INP1 expression is associated with lymph node metastasis of gastric cancer.

Deregulation of genes involved in cell cycle and cell signaling pathways has been described and classified as early events for cyclin D1 and p16 genes or late events for p53, DPC4 and BRCA2

genes in the progression model studies (28). *TP53INP1* is a proapoptotic gene strongly activated during cell stress. Overexpression of TP53INP1 is related to G1 cell cycle arrest and induces p53-mediated cell death (3). In the present study, we showed that the apoptotic index in TP53INP1-positive lesions was higher than that in TP53INP1-negative lesions in gastric cancer tissues by TUNEL detection, indicating that TP53INP1 is related to apoptosis and tumor aggressiveness in gastric carcinoma.

TP53INP is an acute gene that induced by various stress such as UV, heat shock et al (1). Up to now, we have known little function of it. We for the first time revealed impact of the TP53INP1 on the survival in gastric cancer. We showed the survival rate of the TP53INP1-positive cases was longer than that of the TP53INP1-negative cases, especially in the diffuse type.

In conclusion, the present study showed that the reduction of TP53INP1 expression might play roles in gastric carcinogenesis and tumor aggressiveness. Analysis of the TP53INP1 may be useful to evaluate the malignant grade of gastric cancer.

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FIGURE LEGENDS

Figure 1. Immunohistochemical analysis of TP53INP1 expression in gastric carcinoma. **A:** TP53INP1 was strongly expressed in normal gastric mucosa or intestinal metaplasia foci (inset). **B:** TP53INP1 expression decreased in gastric carcinoma (arrow). **C:** Well differentiated tubular carcinoma exhibited moderate alteration of TP53INP1 expression. **D:** Poorly differentiated carcinoma showed weakly staining with TP53INP1 antibody. **E:** P53 expression increased in gastric carcinoma (arrow).

Figure 2. Comparison of TP53INP1 expression (in cell cytoplasm and nucleus) between normal gastric mucosa and gastric cancer tissues (* $p<0.0001$).

Figure 3. Apoptosis analysis in gastric cancer. **A and B:** Representative patterns of TP53INP1-positive (A) and negative (B) carcinoma in TUNEL staining. Arrows indicate TUNEL-positive nuclei (original magnification × 20). **C:** Statistical analysis of apoptotic index in TP53INP1-positive and -negative cancer tissues (* $p<0.0001$).

Figure 4. TP53INP1 expression and patient survival by Kaplan-Meier analysis. **A:** Survival curves for TP53INP1-positive and negative gastric cancers. The 60-months survival rates are 74.7% and 54.9%, respectively. The difference between the values is highly significant ($p=0.0006$). **B:** In well or moderately differentiated adenocarcinoma (intestinal-type), the survival of TP53INP1-positive patients was not significantly different from that of TP53INP1-negative group ($p=0.1110$). **C:** In poorly differentiated adenocarcinoma (diffuse-type), the 60-months patient survival rates are 62.1% and 45.9% for TP53INP1-positive and -negative gastric cancers, respectively. This difference is statistically significant ($p=0.0199$).

Table 1 TP53INP1 expression in gastric cancer

		Non-malignant gastric tissue (n=142)	Gastric cancer (n=142)*	*p* value
TP53INP1	Positive	139 (98%)	91 (64%)	<0.0001
	Negative	3 (2%)	51 (36%)	
P53	Positive	0 (0%)	68 (47.9%)	<0.0001
	Negative	142 (100%)	74 (52.1%)	

*: In gastric cancer the *p* value between TP53INP1 and P53 was 0.006.

Table 2. Correlation between TP53INP1 expression levels and clinicopathologic features in gastric cancer

	TP53INP1-positive (n=91 of 142 patients)	TP53INP1-negative (n=51 of 142 patients)	p value
Age (years)			
<60	31	18	NS
≧60	60	33	
Sex			
Male	68	36	NS
Female	23	15	
Location			
Cardia	20	22	
Body	58	26	0.0193
Antrum	13	3	
Histological type			
Differentiated[a]	62	14	
Undifferentiated[b]	29	37	<0.0001
Tumor invasion			
T1a+T1b	23	4	0.0111
T2+T3+T4	68	47	
Lymph node metastasis			
Positive	5	11	0.0037
Negative	86	40	
Metastasis			
Positive	0	5	0.0024
Negative	91	46	
Lymphatic invasion			
Positive	50	42	0.0010
Negative	41	9	

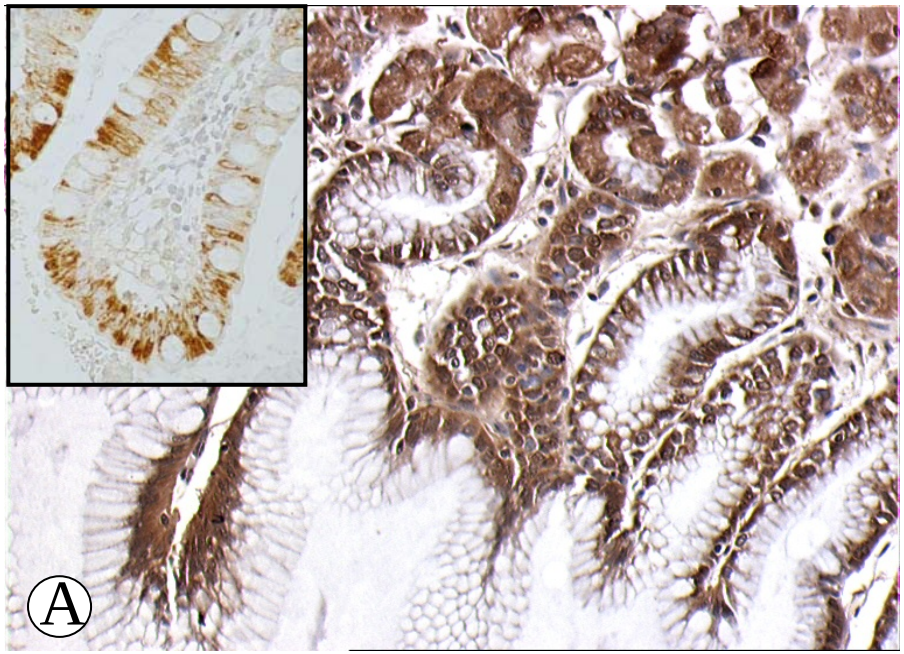
NS: not significant

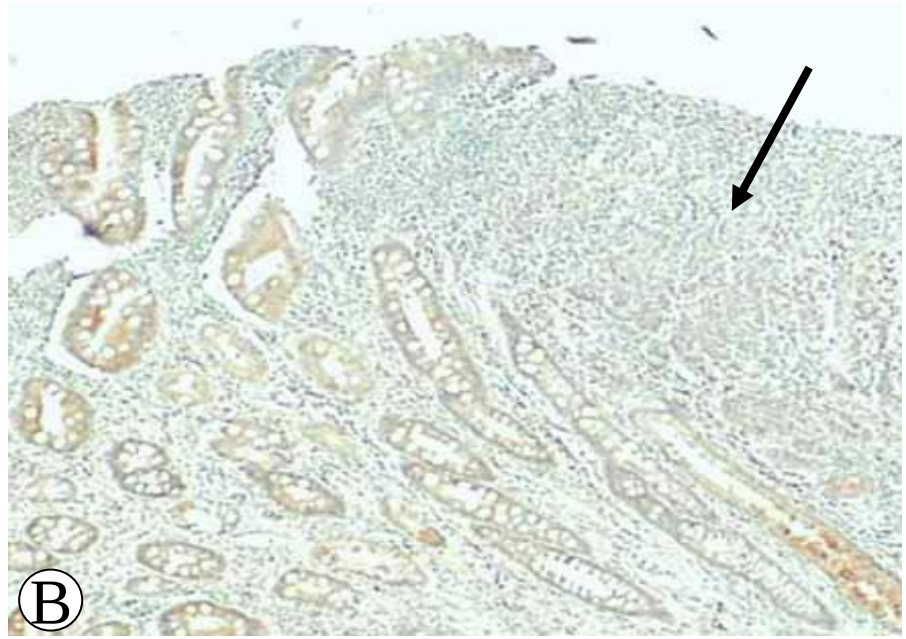
[a]Differentiated type corresponds to well and moderately differentiated tubular and papillar tumors (intestinal type)

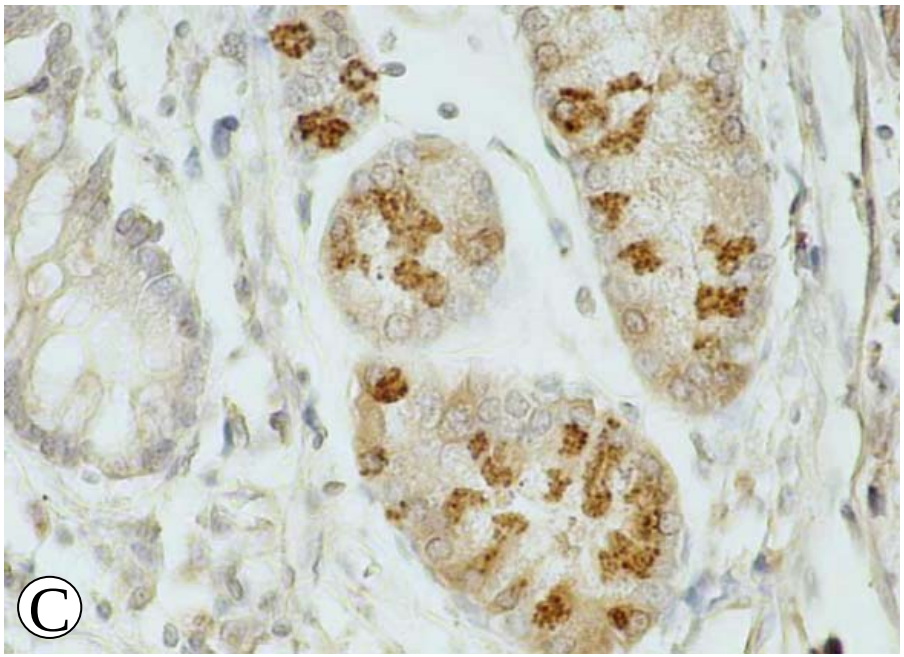
[b]Undifferentiated type includes poorly differentiated and signet-ring cell carcinomas (diffuse type)

Table 3 Multivariate survival analysis using the Cox regression model

factor	Reference	Odds Ratio	*p*
TP53INP1	+ vs -	1.250	0.3680
Age	<60 vs ≥60	1.388	0.1077
Gender	Male vs female	1.158	0.5217
Location	Body+cardia vs autrum	1.253	0.4847
Histological type	Poor vs well+moderately	2.043	0.0026
Tumor invasion	T2+T3+T4 vs T1	1.061	0.8485
Stage	Ⅲ+Ⅳ vs Ⅱ+Ⅰ	1.269	0.2612
Apoptotic index	≤4% vs >4%	2.244	0.0008
Metastasis	+ vs -	18.688	0.0007
Lymphatic invasion	+ vs -	0.721	0.2124
Lymph node invasion	+ vs -	3.121	0.0032







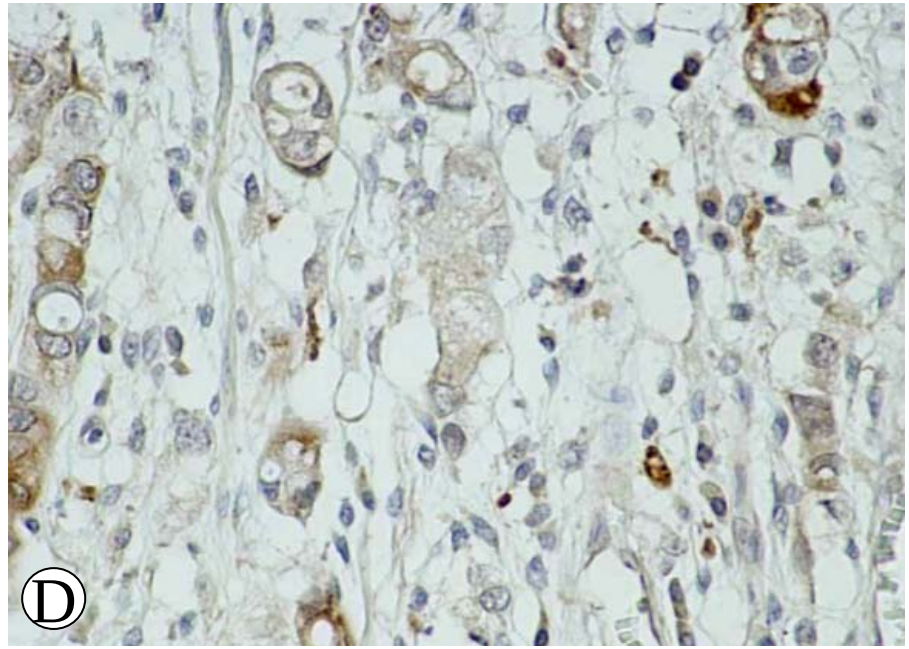


Fig. 1

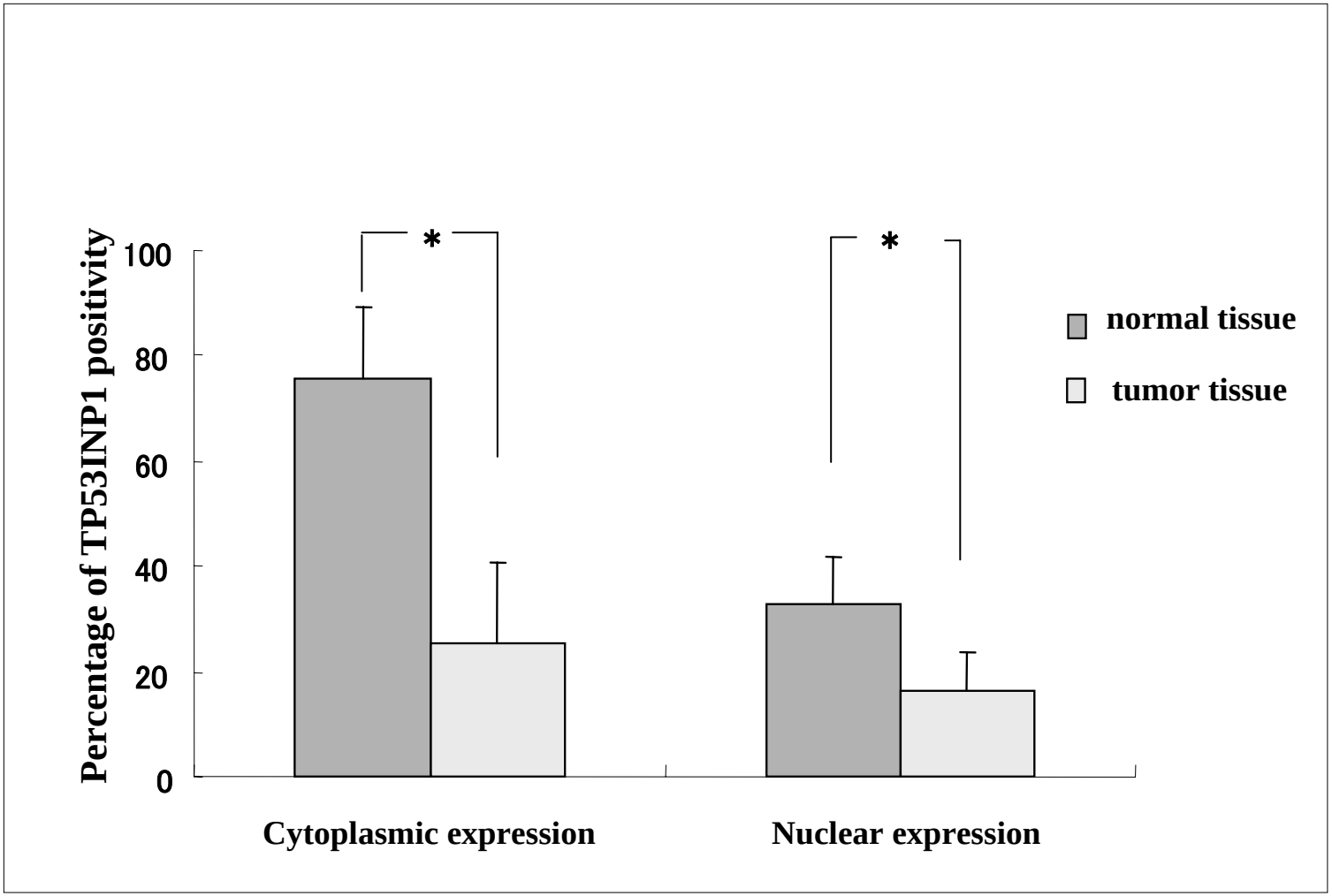
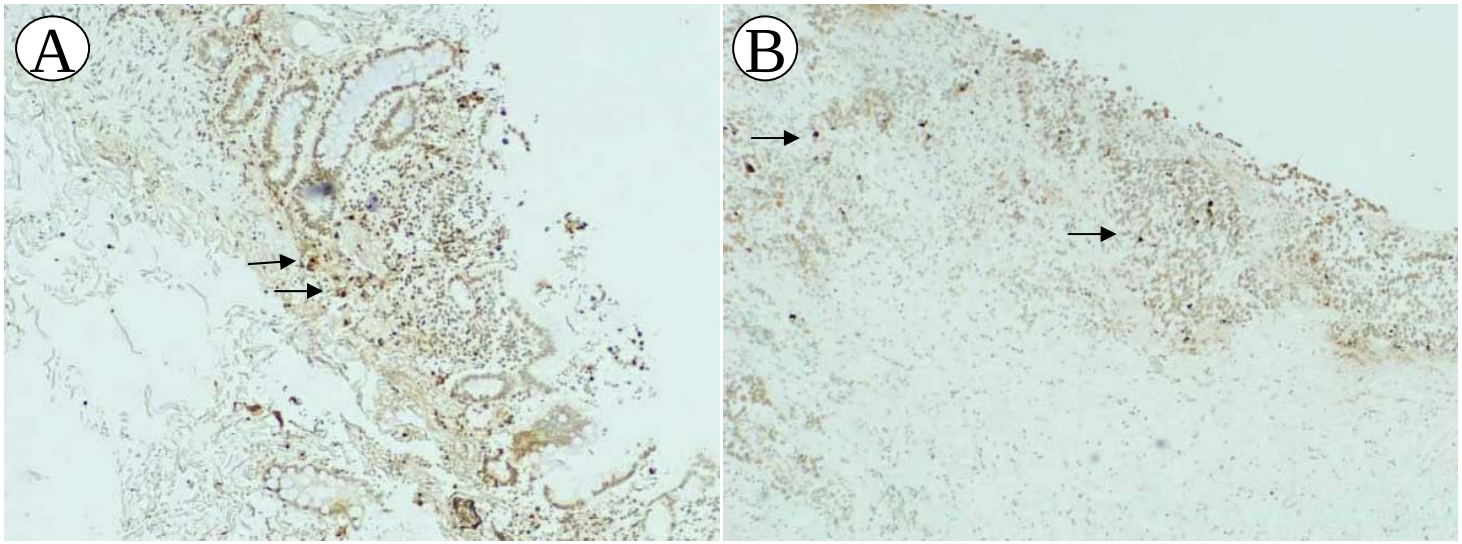

Percentage of TP53INP1 positivity
100
80
60
40
20
0
*
*
normal tissue
tumor tissue
Cytoplasmic expression
Nuclear expression

Fig. 2



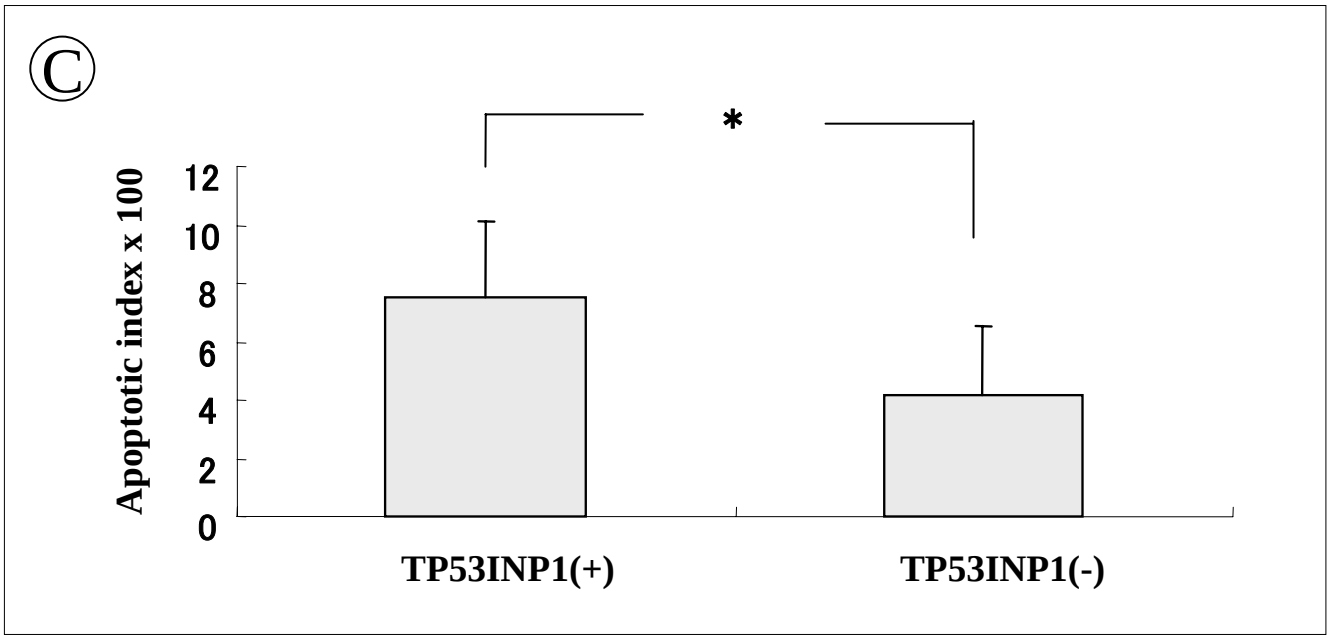
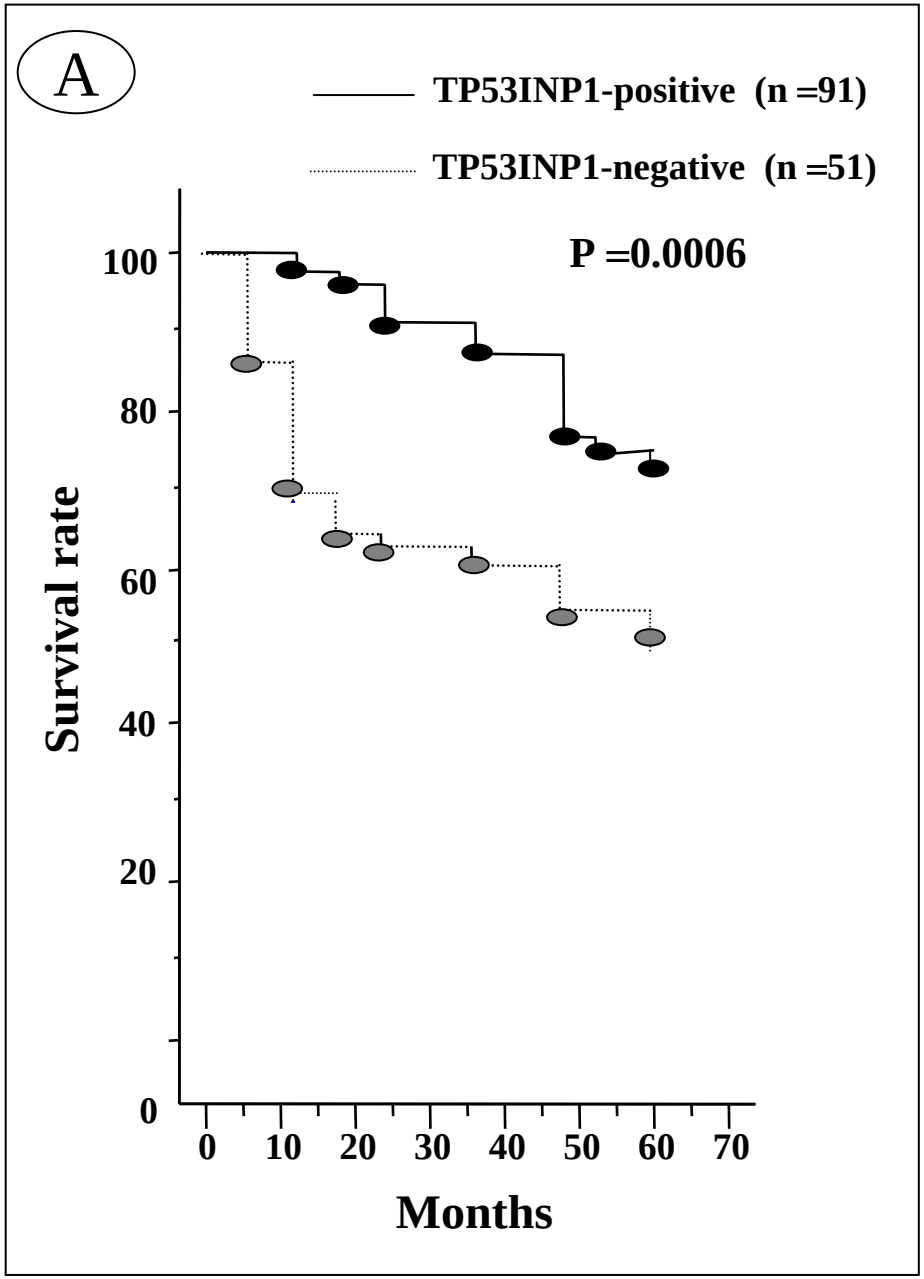


Fig. 3


A
TP53INP1-positive (n =91)
TP53INP1-negative (n =51)
P =0.0006
Survival rate
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Months

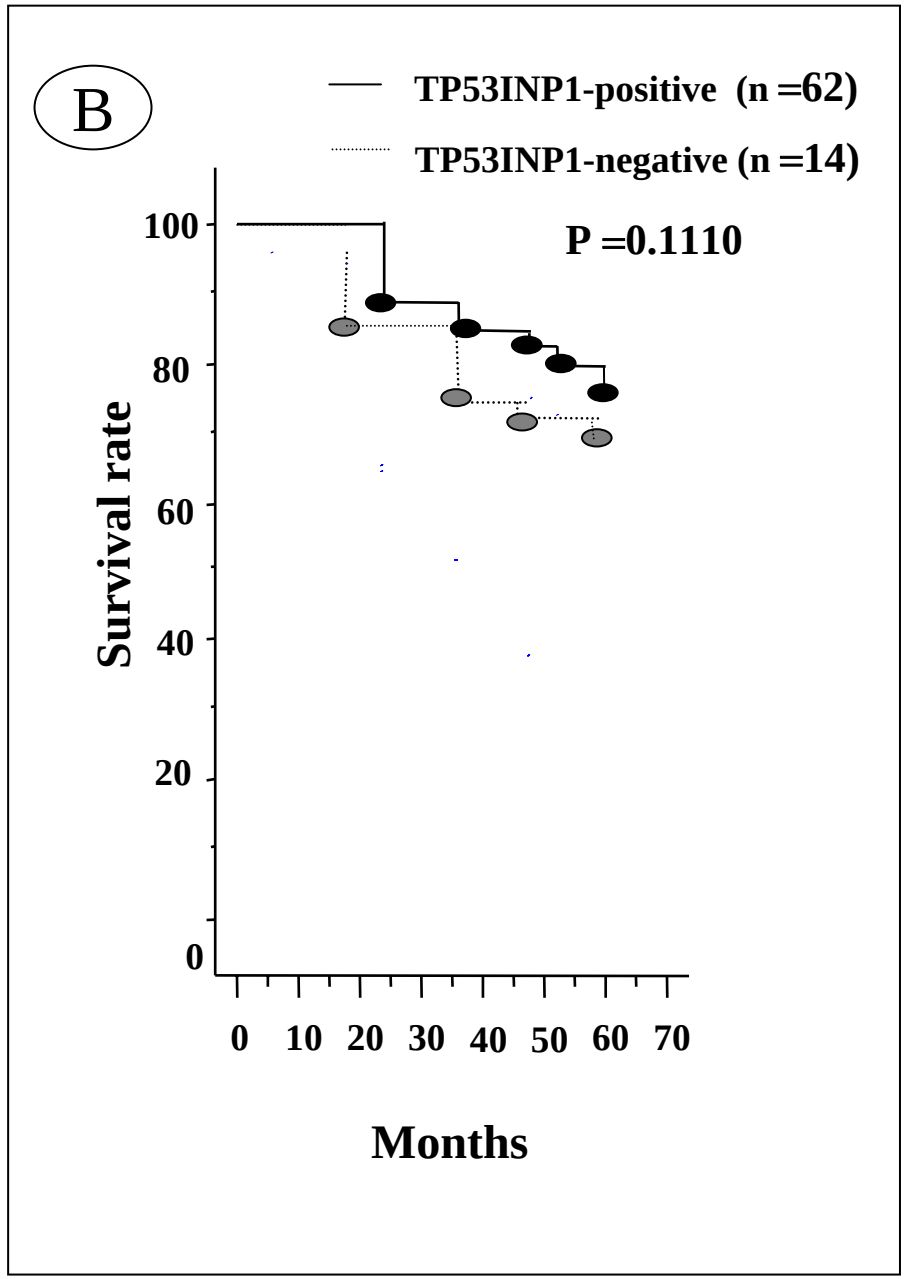

B
TP53INP1-positive (n =62)
TP53INP1-negative (n =14)
P =0.1110
Survival rate
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Months

Fig. 4

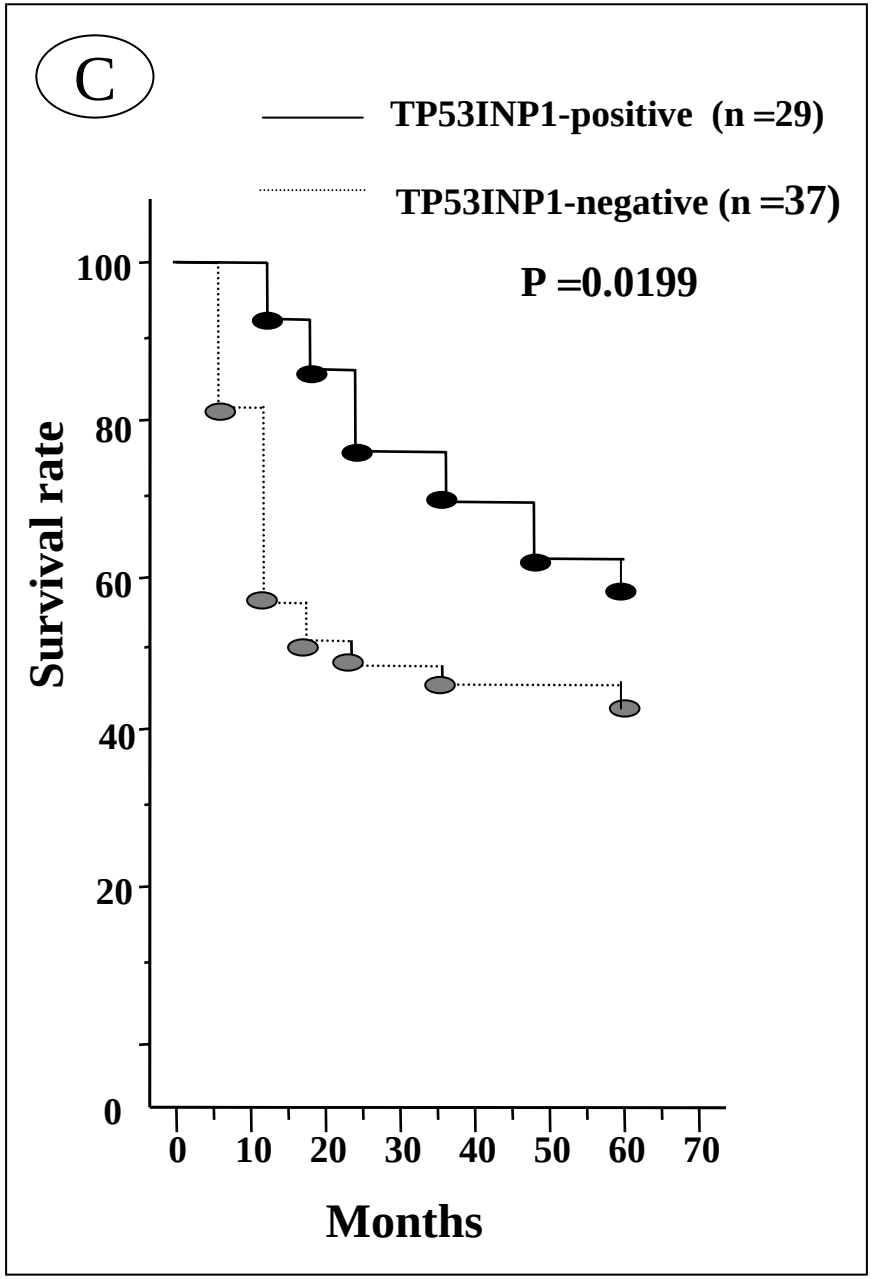

C
TP53INP1-positive (n =29)
TP53INP1-negative (n =37)
P =0.0199
Survival rate
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Months

Fig. 4