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Loss of p21 expression is associated with p53 mutations and increased cell proliferation and p27 expression is associated with apoptosis in maxillary sinus squamous cell carcinoma

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Running title: p21 expression and p53 mutations in maxillary sinus carcinoma

Objective-We have previously reported that p53 mutations, loss of bax expression or decreased spontaneous tumor apoptosis were associated with poor prognoses in maxillary sinus squamous cell carcinoma (SCC) (Cancer 94: 1968-80, 2002). However, whether expression of cell cycle-related protein p21 and p27 and cell proliferation correlate with either p53 status, spontaneous apoptosis or disease prognosis in maxillary sinus SCC has not been evaluated yet.

Patients and Methods-The study group consisted of 70 patients with maxillary sinus SCC who had been studied for apoptosis and p53 status. Tumor biopsy specimens were examined for p21 and p27 using immunohistological methods. The percentage of proliferating cells labeled by anti-Ki-67 mAb was expressed as a Ki-67 index (KI).

Results-Loss of p21 expression correlated with poor tumor cell differentiation (P=0.0234) and p53 mutations (P=0.0072). The KIs in patients without p21 expression and with p53 mutations were significantly higher than those with p21 expression (P=0.0119) and those without p53 mutations (P=0.0048), respectively. On the other hand, patients with p27 expression showed significantly higher apoptotic indices (AI) defined as the number of single-stranded (ss) DNA-positive cells per 1000 tumor cells, than patients without p27 expression (P=0.0012). In 57 patients treated uniformly with preoperative radiochemotherapy followed by total or partial maxillectomy, Kaplan-Meier analysis showed that high KI significantly correlated with decreased disease-free survival (P=0.0341). The p21 expression was closely associated with prolonged

disease-free survival in the group of patients with wild type p53 (P=0.0472).

Conclusions- Loss of p21 expression dependent on the p53 mutation may be associated with a higher tumor cell proliferation, and low p27 expression may be associated with lower levels of spontaneous apoptosis, resulting in poorer prognoses in maxillary sinus SCC. *Key words: apoptosis, cell proliferation, maxillary sinus squamous cell carcinoma, p27, prognosis*

INTRODUCTION

Maxillary sinus squamous cell carcinoma (SCC) is relatively rare in western countries, but occurs frequently in Japan. Understanding the relationships between the biologic and clinical behavior may lead to improvements in predicting the clinical outcome. However, this has not been fully studied in maxillary sinus SCC yet. Previously, we showed that p53 mutations, loss of bax expression, decreased spontaneous apoptosis or low histological effectiveness of radiochemotherapy were significantly associated with poor prognoses in maxillary sinus SCC (1). Furthermore, we found that bax expression was associated with increased spontaneous tumor apoptosis, while decreased spontaneous apoptosis and p53 mutations correlated with low histological effectiveness of radiochemotherapy. These results suggest that high levels of spontaneous apoptosis induced by bax expression may increase the sensitivity of tumor to radiochemotherapy resulting, in good prognoses, while p53 mutations may lead to resistance against radiochemotherapy, thereby resulting in poor prognoses. However, we failed to find the association of p53 status with either bax expression or spontaneous apoptosis, suggesting that the p53 may play a role other than induction of apoptosis in maxillary sinus SCC. Besides induction of apoptosis, p53 protein is known to play an important role in the suppressing cancer cell proliferation at the G1 phase of the cell cycle.

p21 (WAF1/Cip1) and p27 (Kip1) belong to a family of cell cycle regulators

which can bind and inhibit several cyclin-dependent kinases (cdks) (2, 3). Despite their structural and functional similarities, these proteins differ in their mode of activation. A large number of studies demonstrated that the p21 gene product is transcriptionally activated by the p53 in case of DNA damage (WAF1) (4), indicating that p21 is a downstream effector for a p53-dependent pathway. Otherwise, several *in vitro* studies showed that other mechanisms induce p21 expression independently of p53 (5). On the other hand, p27 is activated in response to other extracellular signals such as serum deprivation, contact inhibition and transforming growth factor-ß (6).

As described above, a number of *in vitro* studies have established both p21 and p27 as inhibitory cell cycle proteins and have elucidated their induction mechanisms. However, the clinical roles for p21 and p27 expression in the biological behaviors of human malignancies, especially head and neck SCC, are still controversial. Furthermore, there is little information regarding the expression of these regulatory molecules in primary maxillary sinus SCC. Mutational analysis of the p53 gene has demonstrated a significant correlation between p53 mutations and loss of p21 expression in esophageal SCC (7), but immunohistological analyses showed no correlation between p53 overexpression and p21 expression in laryngeal SCC (8) and head and neck SCC except for maxillary SCC (9). With regard to its correlation with tumor cell proliferation, p21 expression was observed on most tumor cells without BrdU in corporation in head and neck SCC (9), indicating inhibitory role of p21 in the

cell cycle, but contradictory results were reported in laryngeal SCC (10). With regard to correlation with apoptosis, recent immunohistological study showed a significant correlation between p27 expression and increased apoptosis in oral and oropharygeal SCC (11). With regard to correlation with clinical outcomes, some reports have indicated a significant correlation between decreased expression levels of p21 or p27 and poorer prognoses in laryngeal SCC (12), but contradictory results were reported (10). Although a significant correlation between higher proliferation activities of tumor cells and poorer prognoses was reported for head and neck SCC except for maxillary SCC (13), no correlation was reported in laryngeal SCC (14).

In this study, we performed immunohistological analyses for p21 and p27 expression levels and tumor cell proliferation assessed by Ki-67 expression levels. This is the first attempt to investigate correlation of p21 and p27 expression with tumor cell proliferation, apoptosis, p53 mutations, spontaneous apoptosis and disease prognoses in a large cohort of maxillary sinus SCC patients. This study follows diversity from a previous study of the same patient cohort, in which the correlation between tumor apoptosis and p53 status was analyzed (1). Here, we were interested in addressing the following questions: (i) Does p53 play a role in the regulation of cancer cell proliferation in maxillary sinus SCC? (ii) Is p21 expression induced by p53? (iii) Do the expression levels of p21 and p27 correlate with tumor cell proliferation or apoptosis? (iv) Do the expression levels of p21 and p27 and tumor cell proliferation correlate with

the clinical features and the prognoses in the cohort of maxillary sinus SCC patients?

PATIENTS AND METHODS

Patients

The study group consisted of 70 Japanese patients (49 males and 21 females) ranged from 36 to 86 with a median age of 67 years old who were treated for maxillary sinus SCC in the Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College between 1980 and 2000. All patients signed informed consents for therapy and tissue studies, which had received prior approval from the Institutional Review Board. This group had been previously studied for expression of apoptosis-related proteins such as p53, Fas, bax, and bcl-x_L as well as spontaneous tumor cell apoptosis and mutations of the p53 gene in relation to disease prognoses (1).

Clinical features, expression of apoptosis-related proteins and p53 gene status of the patients are listed in Table 1 (1). With regard to expression of apoptosis-related proteins analyzed by immunohistological methods in biopsy tissues at the pretreatment period, p53 was over-expressed in 39 (56%) of 70 patients, Fas was in 20 (29%), bax was in 40 (57%), and bcl-x_L was in 33 (47%). The apoptotic index (AI) value, defined as the number of single stranded (ss)-DNA-positive cells per 1000 tumor cells, ranged from 0.2 to 7.2 with median value of 1.8 (1). Mutations of the p53 gene, which was analyzed by direct sequencing of PCR-amplified products obtained from laser

microdissected tissues, were detected in 20 (29%) patients.

Of 70 patients, 57 patients were treated with preoperative radiochemotherapy followed by total or partial maxillectomy as previously shown by Bandoh et al.(1). Follow-up period of all patients ranged from 2 to 189 months with a median of 61 months. The 5-year disease-free survival rates for all patients were 74.5%; for 13 patients who received radiotherapy alone, these rates were 0%. For 57 patients who underwent preoperative radiochemotherapy followed by total or partial maxillectomy, the follow-up period ranged from 2 to 189 months with a median of 65 months (89 months for surviving patients). Of these 57 patients, 24 (42%) patients had died during follow-up, including 8 (14%) of tumor-related death and 16 (28%) of death from comorbidities. The 5-year disease-free survival rates were 79.7%(1).

Immunohistological staining

The formalin-fixed and paraffin-embedded tissues were obtained from transoral biopsy during the pretreatment period. The tissues were cut in 5μm sections. The sections were deparaffinized with xylene and rehydrated with ethanol. Tissue sections were incubated with 3% hydrogen peroxidase in methanol for 30 minutes. Antigens were retrieved by microwave in 10 mM citrate acid buffer at pH 6.0 for 5 minutes. The following antibodies were used: anti-p21 (WAF1/Cip1) monoclonal antibody (mAb) (clone SX118, diluted 1:50; DAKO A/S, Glostrup, Denmark); anti-p27 (Kip1) mAb (clone

SX53G8, diluted 1:50; DAKO); anti-Ki-67 mAb (clone MIB-1, diluted 1:50; DAKO) for detection of proliferative cells. Tissue sections were sequentially incubated with mAb over night at 4˚C and with peroxidase labeled dextran polymer (EnVision^{+}, DAKO, Carpinteria, CA) for 30 minutes at room temperature. The sections were visualized by immersing the slides in freshly prepared 0.02% diaminobenzidine solution (DAKO) for 10 minutes. The sections were finally counterstained with Lillie-Mayer's hematoxylin and mounted.

Section evaluation

Stained tissue sections were examined microscopically in a coded manner by three of the authors (M.T., Y.H. ,N.B.) who had no knowledge of patients and clinical outcome. More than 5000 tumor cells in at least 5 high power fields were counted, and then the immunoreactive cells of which nuclei were exclusively stained with p21, p27 and Ki-67 were calculated, respectively. The percentage of p21- and p27-positive tumor cells was expressed as a ratio of positive cells to the total number of tumor cells counted. In accordance with previous studies (12, 15, 16), tumors with ≤10% of p21- and p27-positive cells were considered to be negative for p21 and p27 expression, respectively. The Ki-67 index (KI) values were defined as a number of Ki-67 positive cells per 100 tumor cells (14). Sections stained with the anti-mouse IgG1 mAb were used as negative controls. Sections of the tonsil served as positive controls for p21, p27 and Ki-67.

Statistical analysis

Two group comparisons were tested using the Mann-Whitney U test and Fisher's exact test. Results were summarized with their appropriate P-value. The Spearman regression coefficient was used to examine the magnitude of selected association. Disease-free survival (DFS) time was measured from the date of surgical removal of tumor to that of first relapse or that of last follow-up visit. The probability of DFS was calculated by using Kaplan-Meier method and compared using log-rank test. P-values less than 0.05 were considered to be statistically significant.

RESULTS

p21 and p27 expressions and tumor cell proliferation

In 70 patients, the percentage of p21-positive tumor cells in the biopsy tissues during the pretreatment period ranged from 0% to 62% with median value of 9%. Thirty-seven (53%) patients who had ≤10% of p21-postive cells were considered to be negative for p21 expression. The percentage of p27-positive tumor cells ranged from 0% to 42% with median value of 12%. Thirty-one (44%) patients who had ≤10% of p27-postive cells were considered to be negative for p27 expression. Proliferating tumor cells that were positive for Ki-67 were identified in all biopsy specimens and the Ki-67 index (KI) ranged from 2% to 76% with median value of 24%. Loss of p21 expression (≤10%

of p21-positive tumor cells) significantly correlated with poorly differentiated type of tumor (P= 0.0234, Table 2), but p27 expression and the KI did not. Both p21 and p27 expression did not correlate with age, tumor size or lymph node involvement.

Correlation of p21 and p27 expression and tumor cell proliferation with p53 status

As described in our previous report (1), overexpression of p53 protein was found in 39 (56%) of 70 patients and mutation of p53 gene were detected in 20 (29%). There were no significant correlations between the expression levels of p53, p21 and p27. Twenty-nine (88%) of 33 patients with p21 expression (>10% of p21-positive tumor cells) had wild type of p53 gene, while 16 (43%) of 37 patients without p21 expression had mutated p53 gene (P=0.0072, Table 3). Patients without p21 expression had significantly higher KI than those with p21 expression (median:interquartile range=22:16-43, 16:13.5-21.5, respectively; P=0.0119; Fig. 2). Patients with mutated p53 gene showed significantly higher KI also than those with wild type p53 gene (median:interquartile range=24:18-49, 17:12-26, respectively; P=0.0048; Fig. 2). Expression of p53 and p27 did not correlate with KI.

Correlation of p21 and p27 expression and tumor cell proliferation with apoptosis related proteins

Expression of p21 and p27 did not correlate with either expression of Fas, bax or bcl-x_L.

The AI of patients with p27 expression (>10% of p27-positive tumor cells) was significantly higher than that of patients without p27 expression (median:interquartile range=2.2:1.3-4.2, 0.8:0.4-2.4, respectively, P=0.0012, Fig. 3), but the AI was not different between patients with or without p21 expression. The KI was inversely correlated with AI (R= -0.4, P=0.0235, Fig. 4).

Prognosis according to variables

To clarify actual prognostic factors for maxillary sinus SCC, we selected 57 patients treated uniformly with preoperative radiochemotherapy followed by total or partial maxillectomy and analyzed variables. Kaplan-Meier analysis showed that disease-free survival was significantly worse in patients with high KI (≥20) (P=0.0341, Fig. 5a) as compared to patients with low KI. There was no significant difference on disease-free survival according to expression of p53, p21 and p27. However, in the group with normal p53 status (n=25), i.e., without p53 mutations or overexpression, patients with p21 expression (n=12) showed significantly better 5-year disease-free survival rate than patients without p21 expression (n=13) (P=0.0472; Fig. 5b).

DISCUSSION

p21 is known to be a downstream effector of p53-dependent cell cycle regulation. In surgically resected samples of head and neck cancer lesions, it was reported that p21

expression did not correlate with p53 overexpression in head and neck SCC other than maxillary sinus SCC (9) and laryngeal SCC (8). In this study, we found also no correlation in maxillary sinus SCC. These were analyzed by immunohistology. It is generally accepted that mutation analysis of the p53 gene by direct sequencing is more accurate and reliable than immunohistology (17, 18), because immunohistological analysis may consider as positive the non-mutational accumulation of the p53 as a result of interruptions in its normal degradative pathway (19). Our p53 mutation analysis by direct sequencing showed a significant association between p53 mutations and loss of p21 expression in maxillary sinus SCC, as seen in esophageal SCC (7). On the other hand, Nadal et al. (20) reported no association between p53 mutations and p21 expression in 38 patients with laryngeal SCC. Although we cannot resolve this discrepancy, it may be due to differences in the mutation type: frameshift mutations were predominant (6 of 13 mutations) in laryngeal SCC and all tumors with frameshift mutation were p21-positive (20), while missense mutation was predominant (19 of 20 mutations) in maxillary sinus SCC (1). It is possible loss of the p21 expression may be dependent on missense mutations of the p53 gene in some malignancies, including maxillary sinus SCC.

In our series of maxillary SCC, loss of the p21 expression was associated with high activity of the tumor cell proliferation, as assessed by the Ki-67 index. Similarly, Oijen et al. (9) observed that the p21 positive cells did not incorporate BrdU in most

tumors of head and neck SCC, indicating a role of the p21 as a cell cycle inhibitory protein. However, several contradictory results were reported in head and neck SCC excluding maxillary SCC (8) and laryngeal SCC (20). This discrepancy may be possibly explained by whether cell-cycle regulation by p21 expression is p53-dependent or p53-independent. Recently, it has been reported that p21 expression is induced by a p53-independent pathway, as seen in the terminal differentiation of skeletal muscle cells and cells of other lineages (5). In our series of maxillary SCC, high activity of the tumor cell proliferation was also associated with p53 mutations, suggesting p53-dependent pathway via the p21 expression. We have previously found no correlation between p53 mutations and either spontaneous apoptosis or expression of apoptosis-related proteins (1). p53 is more likely to play a role in the regulation of cell proliferation rather than the induction of apoptosis in maxillary SCC.

In this study, we found that patients with p27 expression exhibited higher levels of spontaneous apoptosis than those without p27 expression, indicating a close correlation between p27 expression and apoptosis in maxillary sinus SCC. Although it is still unclear how p27 expression correlates with apoptosis, there are two possibilities considered. Katayose et al. (21) demonstrated that overexpression of p27 using recombinant adenoviral vector induced apoptosis in various human cancer cell lines, suggesting that p27 may play a role in induction of apoptosis. Alternatively it is possible that p27 expression is associated with apoptosis via controlling mechanism for

cyclin D1 activation or cell cycle progression. Han et al. (22) reported that overexpression of cyclin D1 in epithelial cells using a stable transfection method induced growth arrest and apoptosis together with increased expression of p27. In this study, we found that increased spontaneous apoptosis was accompanied by a decrease in cell proliferation activity in maxillary sinus SCC. These findings suggest that both the expression of p27 and activation of apoptosis may be related to mechanisms controlling cell cycle progression.

Prognostic values of p21 and p27 expression as well as proliferation of tumor cells are still controversial in head and neck SCC. In this study, p21 expression did not correlate with prognosis in overall cases. However, in the group of patients with p53 normal status, i.e., without p53 mutation or overexpression, patients with p21 expression showed a significantly prolonged disease free survival, indicating that p21 expression with p53 normal status may be a significant indicator for good prognosis in patients with maxillary sinus SCC. This corresponds to the results previously reported in advanced esophageal carcinoma (15). With regard to correlation between p27 expression and prognosis, p27 expression did not correlate with prognosis in maxillary sinus SCC. On the other hand, we found that high Ki-67 index, i.e., high proliferation activity of tumor cells, significantly correlated with poor prognosis. This agrees with the reports for laryngeal SCC (23, 24) and head and neck SCC except for maxillary SCC (13). Whereas no correlation between the Ki-67 index and prognosis is reported in

laryngeal SCC (14). These contradictory findings suggest that the prognostic values of either p21 and p27 expression or cell proliferation on SCC tissues may vary among tumor sites and/or treatment manners even in the head and neck lesion.

It has been shown that rapidly proliferating tumors usually demonstrate a higher rate at immediate response to radiotherapy than tumors with slow proliferation (25). However, when we examined histological effectiveness of radiochemotherapy in surgically resected specimens obtained from 57 patients with radiochemotherapy followed by maxillectomy, the effectiveness did not correlated with proliferation of tumor cells (data not shown), but it was closely associated with high level of spontaneous tumor apoptosis (1). Therefore, the sensitivity for radiochemotherapy of our regimen is likely to be dependent of spontaneous apoptosis rather than proliferation activity of tumor cells in maxillary sinus SCC. Highly proliferating tumors overcoming cell-cycle controlling system and apoptosis induction system seem to be associated with tumor relapse and/or metastases, resulting in poor prognosis.

Together with our present study and previous study for p53 mutations and apoptosis (1), it was suggested that the p53 mutations, decreased spontaneous apoptosis and increased cell proliferation were significant indicators for worse disease free survival in maxillary sinus SCC. The p53 mutation followed by loss of p21 expression may lead to increased proliferation activity of tumor cells, resulting in poor prognoses, while increased spontaneous apoptosis induced by expression of bax or p27 may

increase sensitivity of radiochemotherapy and down-regulate proliferation activity of tumor cells, resulting in good prognoses.

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Figure legends

Fig. 1 Representative immunohistological features of p21 and 27 protein and proliferative cells in maxillary sinus squamous cell carcinoma. p21 (a) and p27 (b) protein showed nuclear staining. (c) Proliferative cells stained by anti-Ki-67 mAb also showed nuclear staining. Magnification: (a,b and c) x250.

Fig. 2 Ki-57 index (KI) between patients with or without p21 expression and p53 mutation. (a) Patients without p21 expression had significantly higher KI than patients with p21 expression. (b) Patients with mutated p53 gene showed significantly higher KI than patients with wild type of p53 gene. The median values are expressed as a short bar. The Mann-Whitney U test was used to determine P-values.

Fig. 3 Apoptotic index (AI) between patients with or without p27 expression. The AI of patients with p27 expression was significantly higher than that of patients without p27 expression. The median values are expressed as a short bar. The Mann-Whitney U test was used to determine the P-value.

Fig. 4 Correlation between Apoptotic index (AI) and Ki-57 index (KI). The KI was inversely correlated with AI. The Spearman coefficient was used to determine R and P-values.

Fig. 5 Disease free survival according Ki-67 index (KI) and p21 expression without p53 mutation or expression in maxillary sinus SCC who were treated with preoperative radiochemotherapy followed by total or partial maxillectomy. (a) The patients with high

KI showed significantly worse disease-free survival than the patients with low KI. (b) The patients without p21 expression showed significantly worse disease-free survival than patients with p21 expression in the group without p53 expression. Kaplan-Meier method and log-rank test were used for these analyses.

Table I

Clinical features, p53 gene status and expression of apoptosis-related proteins in 70 patients with maxillary sinus squamous cell carcinoma

Variable		Number (%)
Gender	Male	49 (70%)
	Female	21 (30%)
Age (years)	<65	29 (41%)
	≥65	41 (59%)
Tumor extension	T2	8 (11%)
	T3	33 (47%)
	T4	29 (42%)
Lymph node	N0	63 (90%)
	N1	7 (10%)
Clinical stage	II	8 (11%)
	III	33 (47%)
	IVA	28 (41%)
	IVC	1 (1%)
Tumor differenti	well	29 (42%)
	moderately	24 (34%)
	poorly	17 (24%)
p53 gene	Wild type	50 (71%)
	Mutated ty	20 (29%)
p53 protein	negative	31 (44%)
	positive	39 (56%)
Fas	negative	50 (71%)
	positive	20 (29%)
bax	negative	30 (43%)
	positive	40 (57%)
bcl-x_L	negative	36 (53%)
	positive	34 (47%)
Apoptotic index	<2	37 (53%)
	≥2	33 (47%)

TNM staging was performed according to 1997 UICC system. p53 gene status and expression of apoptosis-related proteins have been analyzed elsewhere (1).

Table II
Correlation between expression of p21 and p27 protein and tumor differentiation

		p21 protein			p27 protein		
		positive (n=33)	negative (n=37)		positive (n=39)	negative (n=31)	
		Number (%)	Number (%)	P*	Number (%)	Number (%)	P*
Tumor	well/moderately (n=53)	29 (55%)	24 (45%)	0.0234	31 (58%)	22 (42%)	0.34
differentiation	poorly (n=17)	4 (24%)	13 (76%)		8(47%)	9 (53%)	

*P-values were determined by the Fisher's exact test

Table III

Correlation among p53 mutation, expresion of p21, p27 and p53 protein in 70 patients with maxillary sinus squamous cell carcinoma

Variables		p53 gene			p53 protein			p27 protein	
		wild type (n=50)	nutated type (n=20)		negative (n=31)	positive (n=39)		negative (n=31)	positive (n=39)
		Number (%)	Number (%)	P*	Number (%)	Number (%)	P*	Number (%)	Number (%)
p21 protei	negative (n=37)	21 (57%)	16 (43%)	0.01	15 (41%)	22 (59%)	0.63	15 (41%)	22 (59%)
	positive (n=33)	29 (88%)	4 (12%)		16 (48%)	17 (52%)		16 (48%)	17 (52%)
p27 protei	negative (n=31)	23 (74%)	8 (26%)	0.79	16 (52%)	15 (48%)	0.72		
	positive (n=39)	27 (69%)	12 (31%)		15 (38%)	24 (62%)			

*P-values were determined by the Fisher's exact test

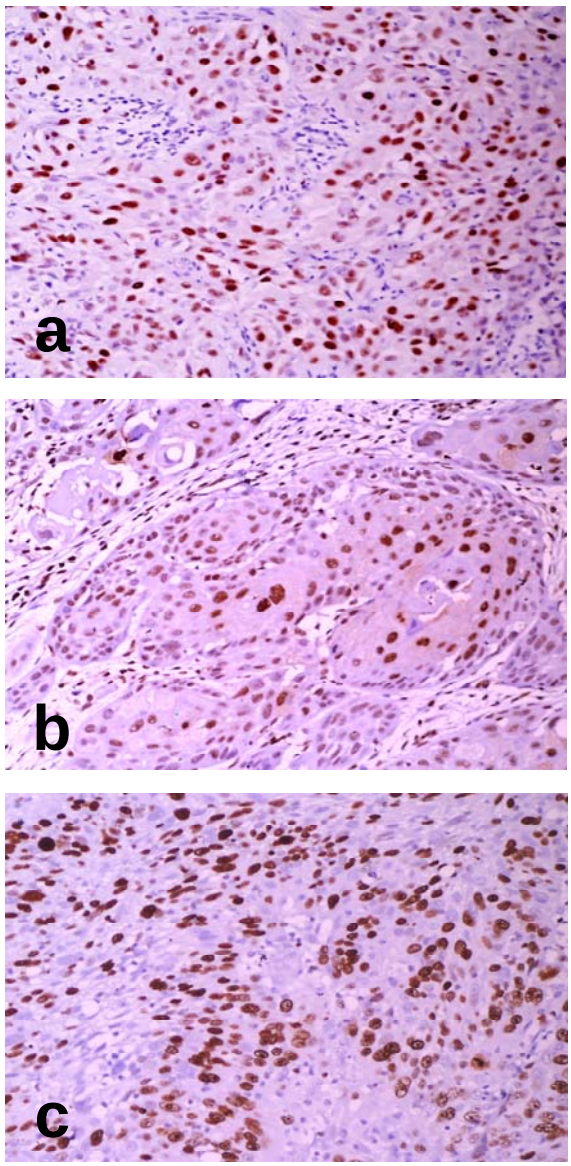

a
b
c

Fig. 2 a/b

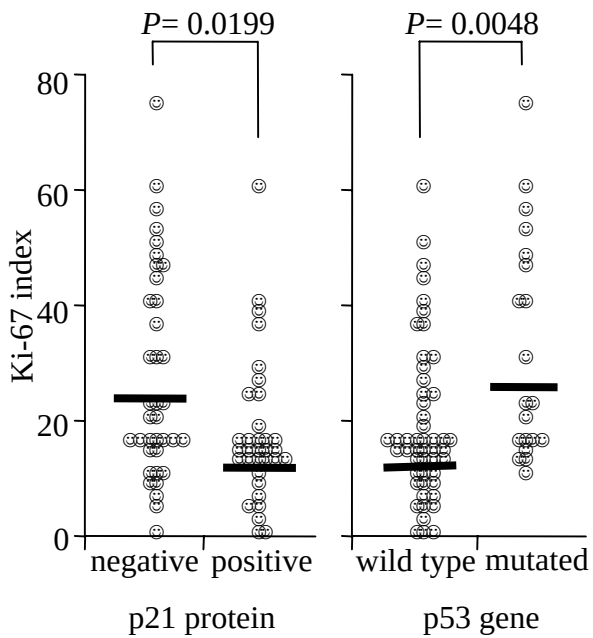

P= 0.0199
P= 0.0048
80
60
40
20
0
Ki-67 index
negative
positive
wild type
mutated
p21 protein
p53 gene

Fig. 3

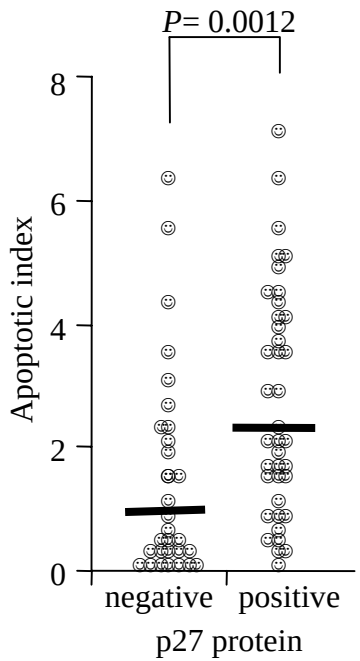

P= 0.0012
Apoptotic index
8
6
4
2
0
negative
positive
p27 protein

Fig. 4

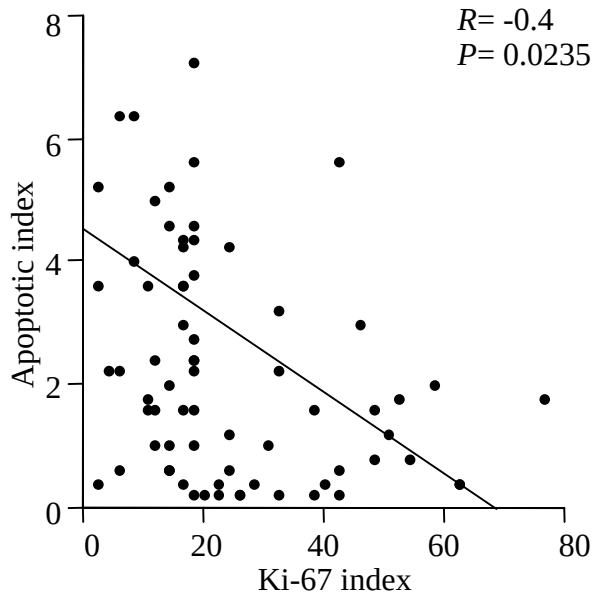
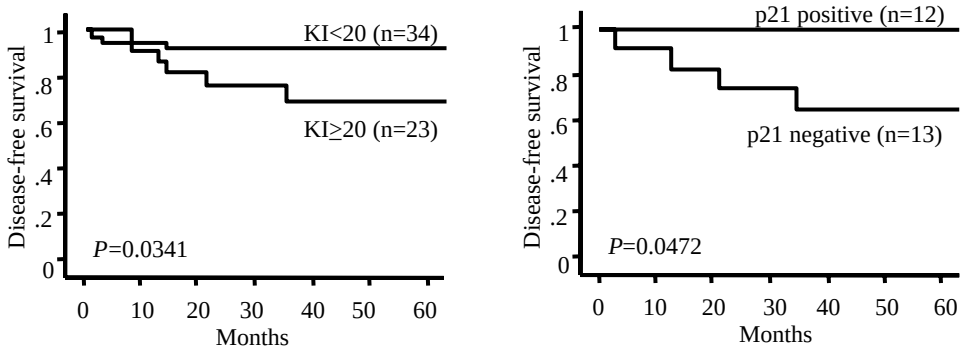


Fig. 5 a/b


Disease-free survival
1
.8
.6
.4
.2
0
KI<20 (n=34)
KI≥20 (n=23)
P=0.0341
0
10
20
30
40
50
60
Months
Disease-free survival
1
.8
.6
.4
.2
0
p21 positive (n=12)
p21 negative (n=13)
P=0.0472
0
10
20
30
40
50
60
Months