Jpn J Clin Oncol 2003;33(7)346–352

p27 Labeling Index and Proliferation in Gastrointestinal Stromal Tumors: Correlations with Clinicopathologic Factors and Recurrence

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Received February 21, 2003; accepted June 15, 2003

Background: Low expression of p27, a cyclin-dependent kinase inhibitor, has recently been reported to be associated with poor prognosis of many tumors. Moreover, an inverse relationship between p27 expression and proliferation was also noted. Although accumulating data indicate a correlation between high proliferation rate and aggressive behavior of gastrointestinal stromal tumors, no conclusive correlation between p27 expression and either tumor recurrence or proliferation has been established yet in this disease. The aim of this study was to immunohistochemically investigate the association of p27 expression (as measured by the p27 labeling index: p27LI) with recurrence and proliferation (as measured by the Ki-67 labeling index: KLI) in gastrointestinal stromal tumors.

Methods: p27LI and KLI were investigated in tumor specimens from 50 patients diagnosed with gastrointestinal stromal tumors. Quantitative evaluations of p27LI and KLI were performed on tissue sections stained immunohistochemically with anti-p27 and anti-Ki-67 monoclonal antibodies.

Results: Both p27LI and KLI were associated with the presence of recurrence and high mitotic index. A significant inverse correlation was noted between p27LI and KLI ($r = -0.82$). The recurrence-free survival time was significantly shorter in patients with high KLI (≥10%), low p27LI (<18%), mitosis (≥4), and larger tumor size (≥5.2cm). Multivariate analysis indicated that p27LI, KLI, and mitosis were independent predictors of recurrence-free survival.

Conclusions: These findings support the view that p27LI is a reliable marker in predicting recurrence and recurrence-free survival in gastrointestinal stromal tumors. Moreover, the concordance of high KLI together with low p27LI, and vice versa, might allow us to measure the aggressiveness of these tumors.

Key words: p27 – proliferation – Ki-67 – gastrointestinal stromal tumors – recurrence

INTRODUCTION

The loss of regulatory control in the cell cycle is an important determinant of malignant transformation and is strongly related to the progression and aggressiveness of many human cancers (1,2). In the past several years, various studies have shown that progression of the cell cycle depends on complex interactions among multiple cell cycle regulators (3–5). Cell cycle transitions are governed by sequential activation and inactivation of cyclin-dependent kinases (cdks) (3–4). Their activities are regulated in turn by a series of positive effectors, the cyclins (2–5). The cyclins contribute to cell cycle control by forming complexes with cdks (5). The activation of the cdks/cyclin complex is controlled by cdks inhibitors, which regulate the cell cycle (2–4,6).

p27, a major inhibitor of the cdks, is a nuclear phosphoprotein belonging to the Kip family of cdks inhibitors (7–11). In physiological conditions the expression of p27 is highest in quiescent cells and declines when cells proliferate in response to mitogenic signals such as growth factors and cytokines, suggesting that it also plays a role in maintaining cells in G0 (12–14). The overexpression of p27 protein in mammalian cells induces a G1 block of the cell cycle. p27 may act as a tumor suppressor and several reports on solid neoplasms of the gastrointestinal system suggest that loss of or low levels of p27 expression is associated with aggressive behavior and poor prognosis (15–17). Moreover, an inverse relationship between p27 expression and proliferation was noted in certain tumors (18,19).

Gastrointestinal stromal tumors (GIST) are a heterogeneous group of neoplasms with biological behaviors that are difficult

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Abbreviations: GIST, Gastrointestinal stromal tumors; P27LI, p27 labeling index; KLI, Ki-67 labeling index; MI, mitotic index

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Table 1. Correlation between clinicopathological factors and KLI and p27LI

	↓KLI (*n*=28)	↑KLI (*n*=22)	*P* value*	↓p27LI (*n*=24)	↑p27LI (*n*=26)	*P* value*
Age						
<46 years	12	12	ns	11	13	ns
≥46 years	16	10	ns	13	13	ns
Gender						
Male	17	12	ns	13	16	ns
Female	11	10	ns	11	10	ns
Localization						
Stomach	13	8	ns	9	12	ns
Small bowel	10	9	ns	9	12	ns
Large bowel	5	5	ns	5	5	ns
Tumor diameter						
<5.2 cm	12	11	ns	13	10	ns
≥5.2 cm	16	11	ns	11	16	ns
Histological subtype						
Epitheloid	8	5	ns	6	7	ns
Spindle cell	12	12	ns	14	10	ns
Epitheloid and spindle cell (mixed)	8	5	ns	4	9	ns
Immunohistochemisty						
CD34						
Positive	24	19	ns	21	22	ns
Negative	4	3	ns	3	4	ns
SMA						
Positive	7	5	ns	18	20	ns
Negative	21	17	ns	18	20	ns
Mitosis						
<4/50HPF	19	8	0.02	8	19	0.005
≥4/50HPF	9	14	0.02	16	7	0.005
Cellularity						
Low	13	14	ns	13	14	ns
High	15	8	ns	11	12	ns
Necrosis						
Absent	18	12	ns	13	17	ns
Present	10	10	ns	11	9	ns
Recurrence						
Absent	21	9	0.01	9	21	0.002
Present	7	13	0.01	15	5	0.002

ns, not significant; **P* values were evaluated based on Chi-square test.
↓KLI, Low KLI (<10%); ↑KLI, High KLI (≥10%); ↓P27LI, Low P27LI (<18%); ↑P27LI, High P27LI (≥18%)

to predict. Complete surgical removal remains the best current therapy for GIST; but, even major resections are associated with recurrence in a great majority of cases (20).

Although high mitotic count and larger tumor size are generally accepted as the best indicator of malignancy, the definitions proposed for the cut-off point to distinguish GIST based on size and mitotic count vary widely (21,22). Currently, many new parameters are used as a supplement for mitotic count and tumor size to better assess the biological behavior of GIST. Measurement of proliferative index is among them. In some studies, high proliferative index was found to be a reliable factor in predicting clinically aggressive behaviors of GIST (23–28). However, the clinicopathologic significance of p27 expression and its association with recurrence and proliferation in these tumors are unknown.

The objective of this study was to immunohistochemically investigate the association of the p27 labeling index (p27LI) with recurrence in GIST and to determine its correlation with

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Table 2. Primary antibodies used in this study and their clones, dilutions, pretreatment modalities and their commercial sources

Antibody	Clone	Dilution	Pretreatment	Source
CD117	Polyclonal	1:400	MW*	Santa-Cruz Biotechnology
CD34	QBEND/10	1:25	MW	Dako
SMA†	1A4	1:25	MW	Dako
Desmin	D33	1:50	MW	Dako
S100 protein	Polyclonal	1:1600	None	Dako
Ki-67	MIB-1	1:80	MW	Immunotech
p27	SX53G8	1:50	MW	Dako

*MW, microwave; †SMA, α-smooth muscle actin.

proliferation (as expressed by the Ki-67 labeling index, KLI) and clinicopathological parameters.

PATIENTS AND METHODS

Selection of Patients

Sixty-eight case records of patients treated for the primary manifestation of a mesenchymal tumor of the gastrointestinal system at the Department of Surgery, Akdeniz University, Antalya, from 1989 to 2002 were retrieved from the files. After reviewing the sections from the representative formalin-fixed tissue blocks by avidin-biotin peroxidase complex detection technique for CD117, CD34, smooth muscle actin, desmin, and S100, 56 cases were diagnosed as GIST according to the definition, which was: a cellular spindle cell, epitheloid or pleomorphic mesenchymal tumor of the gastrointestinal system that expresses CD117 (22,29). In addition, CD34 was positive in 43 cases. Reactivity for smooth muscle actin (SMA) was seen in 12 cases. Desmin and S100 were negative in all cases (Table 1).

All patients were treated by complete surgical resection and no residual tumors were detected. In 19 of these cases, complete resection necessitated resection of adjacent organs to ensure adequate margins.

Recurrence, the end-point of this study, was defined as the reappearance of tumor at the initial site of the primary tumor; therefore, two cases with metastasis and four cases without follow-up information were excluded from the study group. Thirty patients (60%) without recurrence and with a mean survival duration of 45 months (range 32–62), and 20 patients (40%) who experienced recurrence in 2 to 45 months (mean, 18), were included in our study.

Distribution of clinicopathologic characteristics of the patients are summarized in Table 1.

Immunostaining for Ki-67 and p27 was performed using the avidin-biotin peroxidase complex detection technique on 4 μm thick tissue sections from the representative tissue blocks embedded in paraffin. Diaminobenzidine containing hydrogen peroxide was used as the chromogen and all slides were counterstained with haematoxylin. The dilutions of the primary antibodies, their sources and epitope retrieval modalities are listed in Table 2.

A pathologist who had no knowledge about the clinicopathologic data interpreted the slides for mitotic index (MI) KLI and p27LI.

MI was obtained by counting mitotic figures in 50 high power fields and expressing the results as the number of mitoses per 50 HPF. KLI and p27LI were assessed in areas with the highest nuclear labeling density. In each case positive and negative nuclei were counted at ×400 magnification. KLI and p27LI were calculated as the percentage of positive cells relative to the total number of cells counted.

Chi-square test was used to compare frequencies. The difference in numerical data between groups was analyzed by using Mann–Whitney U test. Correlations among various parameters were tested by calculating Spearman's correlation coefficient. Univariate and multivariate survival analysis were performed by using Kaplan–Meier method and Cox regression analysis, respectively. A significance level of 0.05 was used throughout the analysis.

RESULTS

In all positive cases, Ki-67 and p27 immunostaining was diffuse or granular and was confined to the nucleus (Figs 1, 2).

Table 3 shows mean, median, standard deviation, and range of values for tumor diameter, MI, KLI and p27LI. Cases were divided into two groups according to the median values of these parameters for further analysis.

No correlation between KLI, p27LI and clinicopathologic factors such as age, gender, localization, tumor diameter, histological subtype, cellularity, necrosis, CD34 and SMA positivity was detected (Table 1). However, higher MI and the presence of recurrence were more frequent in tumors with low p27LI and high KLI ($P < 0.05$). According to Mann–Whitney U test, the mean KLI of tumors with ≥4 MI was 24.3%, which is significantly higher than that of KLI tumors with <4 MI (8.9%) ($P < 0.01$). In contrast, tumors with ≥4 MI had significantly lower p27LI than those with <4 MI ($P < 0.01$) (Table 4). When mean KLI and p27LI were evaluated in tumors with or without recurrence, tumors with recurrence had significantly

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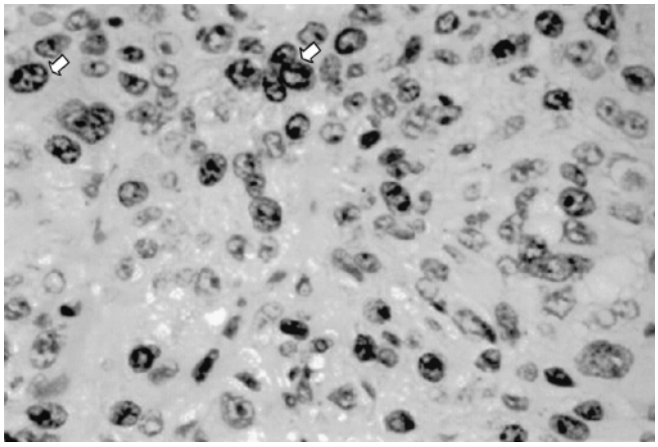


Figure 1. Immunostaining for Ki-67 in GIST. Tumor cell nuclei are positive (Immunoperoxidase stain, original magnification ×400).

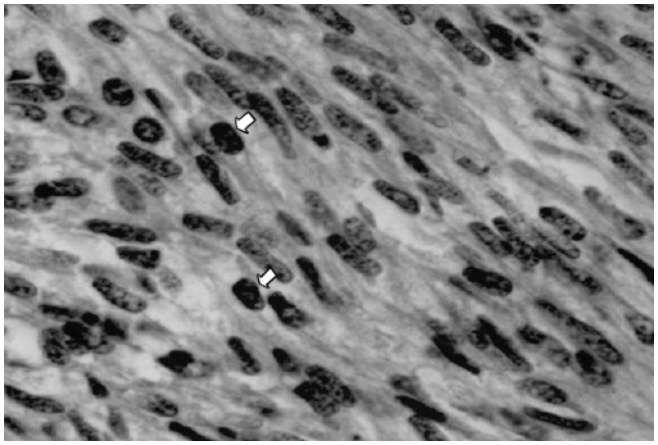


Figure 2. Immunostaining for p27 in GIST. Tumor cell nuclei are positive (Immunoperoxidase stain, original magnification ×400).

higher KLI and lower p27LI than those without (P<0.05) (Table 4).

Spearman correlation test revealed a strong positive correlation between MI, KLI (r = 0.62) and an inverse relationship between p27LI and both MI (r = –0.69) and KLI, respectively (r = –0.82) (P < 0.01).

Univariate analysis based on the log-rank test revealed that the recurrence-free survival of patients in the high MI group (21%) was significantly shorter than in the low MI group (55%) (P < 0.001) (Fig. 3a). Similarly, in the high KLI group

Table 3. Tumor diameter, MI, KLI and p27LI in all cases

	Tumor diameter (cm)	MI	KLI	p27LI
Mean	5.7	4.8	14.6	22.7
Median	5	4	10	18
Standard Deviation	2.4	2.7	8.3	12.7
Minimum	1	1	0	0
Maximum	15	18	46	72

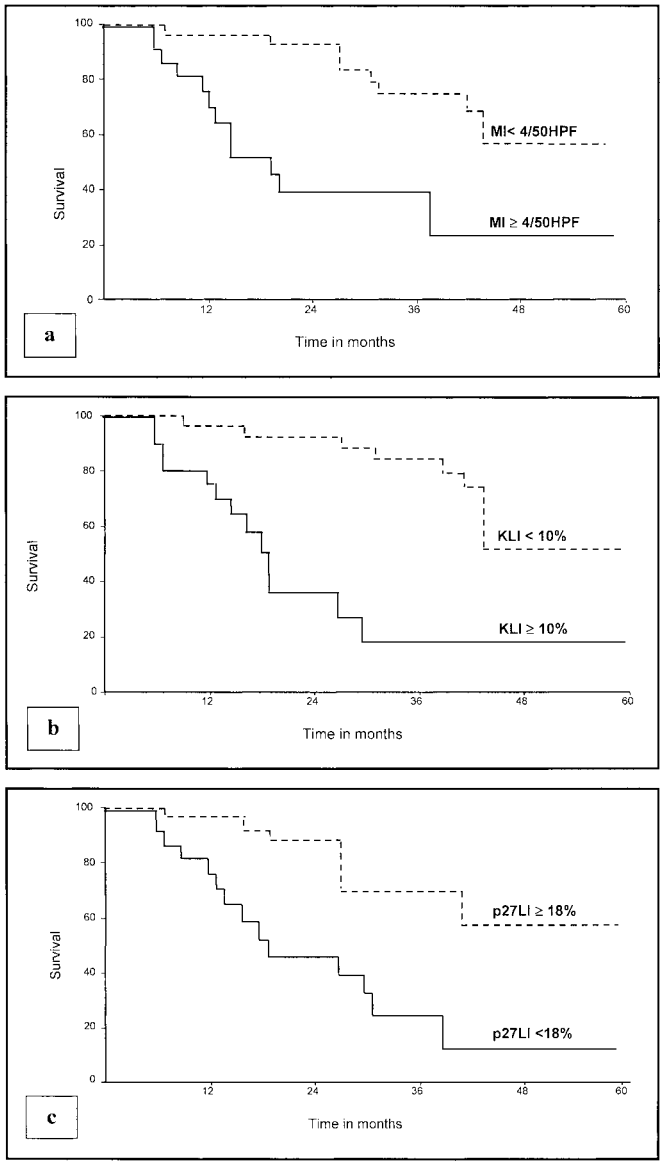


Figure 3. Survival curves of patients with GIST, according to MI (a), KLI (b) and p27LI (c). The survival curves differed a) between patients with tumors showing ≥4 MI and patients with tumors showing <4 MI (P < 0.001); b) between patients with tumors showing ≥10% KLI and patients with tumors showing <10% KLI (P < 0.001); and c) between patients with tumors showing <18% of p27LI and patients with tumors showing ≥18% p27LI (P < 0.001).

the recurrence-free survival was significantly shorter than in the low KLI group. The survival rates of the high KLI and low KLI groups were 18% and 51%, respectively (P < 0.001) (Fig. 3b). However recurrence-free survival rates in patients with low p27LI was 12%, which was significantly lower than that of patients with high p27LI (59%) (P < 0.001) (Fig. 3c). In addition to MI, KLI, and p27LI, the recurrence-free survival time of the patients was affected by tumor size. The difference in the recurrence-free survival of patients with larger tumor size (54%) and smaller tumor size (23%) was statistically significant (P < 0.001). In multivariate analysis with covariates that showed statistical significance in the univariate analysis, MI, KLI, and p27LI were found to be independent prognostic

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Table 4. Distributions of KLI and p27LI values according to MI and recurrence

	KLI				p27LI			
	Mean	Median	SD	Ranges	Mean	Median	SD	Ranges
Mitosis*								
<4/50HPF	8.9	7	4.9	0–31	30.2	39	16.3	12–72
≥4/50HPF	24.3	14	13.4	0–46	10.5	18	13.5	0–59
Recurrence†								
Absent	10.7	11	4.5	0–32	29.4	23	14.9	14–72
Present	19.4	17	9.9	0–46	14.6	16	6.7	0–52

Mann–Whitney U test, *$P < 0.01$; †$P < 0.05$

Table 5. Multivariate analysis indicating independent factors correlated with disease free survival period in GIST

Parameters	Regression coefficient	Standard error	95% CI*	Relative risk	*P* value
MI	1.103	0.480	1.176–7.718	3.012	0.02
KLI	1.837	0.335	2.617–9.479	4.034	0.003
p27LI	–1.753	0.476	0.046–0.652	0.17	0.009

*CI, Confidence interval.

factors (Table 5). However, in the same analysis, tumor size did not reach significance ($P = 0.629$).

DISCUSSION

p27, a cell cycle inhibitor, binds to a wide variety of cyclin/cdk complex, inhibits their kinase activity, and thus blocks the cell cycle (7–11). Therefore, the loss of p27 expression is thought to reflect rapid tumor growth, and p27 expression is often deregulated in human tumors. Recent studies have shown the association of absent or low p27 protein expression with tumor progression and aggressiveness (15–17,30–32). To our knowledge, however, p27 expression has not been evaluated in GIST. In this study, we observed that p27LI might help to better predict the recurrence risk of these tumors. In fact, in our cases we could show that the p27 expression is directly correlated to recurrence-free survival. Tumors with low p27LI were more frequent in patients who experienced recurrence than those without. In our series, the prognosis of patients in the high p27LI group was more favorable than those in the low p27LI group ($P < 0{,}005$). Furthermore, multivariate analysis showed that p27LI was a significant and independent prognostic factor in predicting the probability of recurrence-free survival. Although p27LI is not the only factor responsible for proliferation, these data support the previous observations on deregulated expression of p27 in malignant tumors and lead us to speculate that the level of p27LI expression in GIST could be an important parameter in estimating the aggressiveness of these tumors.

It is well established that cell proliferation kinetics are important in predicting the prognosis of various tumors. Numerous studies demonstrated that the cell proliferation is associated with clinical outcome in a number of malignancies, including GIST (23–28). The results of our investigation confirm the association of high proliferation indices in GIST with the higher risk of recurrence. Our study further revealed that both KLI and MI were associated with recurrence-free survival of patients with GIST. It should be noted, however, that data from various sources do not reach an agreement regarding the actual numerical values in the assessment of proliferative indices by means of immunohistochemistry in GIST. It has been pointed out that the use of different antibodies to define proliferating cells is an important limitation in predicting the exact proliferative rate of many organ tumors (28). Some GIST studies used proliferating cell nuclear antigen to assess the cell growth fraction, but the significance of its reactivity is questionable (23,24,33). We therefore used Ki-67 immunohistochemistry to estimate the cell proliferation activity. Ki-67 is a nuclear protein expressed exclusively in cycling cells; but, it is not present in quiescent cells (G0) (34,35). In our study, the mean KLI value of the whole group was 14.6%. In recent studies, mean values of KLI in GIST showed differences among researchers, ranging from 4% to 22% (25–28). We suggest that different methodologies in the assessment of proliferation and inter-observer variations could also contribute to this discordance between the actual numerical values (26,27). However, these do not undermine the significance of our findings that showed a close correlation between KLI, MI and aggressive behavior in GIST. Our data support the proposition that evaluation of proliferation by means of KLI or MI could be valuable in predicting the malignant potential of these tumors.

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Recently, the highly proliferative activity as measured by Ki-67 antigen has been shown to correlate with a reduced p27 expression in lymphomas, endocrine tumors, malignant mesothelioma, synovial sarcoma, and bile duct carcinomas (18,19,36–38). However, the relation between proliferation and p27LI was not previously analyzed in GIST. Therefore, we carried out the present study to also investigate the association of proliferative activity (as measured by the expression of Ki-67 and mitosis) with alterations of p27LI in these tumors. Our results revealed that the p27LI was inversely correlated with KLI and MI ($P < 0.001$). All these findings suggest that decreased expression of p27 may play important roles in the subsequently increased cell turnover that is related with the malignant potential of GISTs. Moreover, the concordance of these parameters (high KLI or MI together with low p27LI, and vice versa) allows us to identify the biological behavior of these tumors.

In a recent study, the presence of a TGF-α/EGFR autocrine loop has been reported in GISTs (39). It was shown that p27 might provide an important link in pathways that connect EGFR and TGF-α mediated mitogenic signals to the cell cycle in the G1-S transition (40). From this point of view, it would be of interest to elucidate the relationship between p27 expression and EGFR and TGF-α expression in GISTs.

Our objective in this study was to investigate the association of p27LI with recurrence in GIST and to determine its correlation between proliferation and clinicopathological parameters. Although our data remains to be further established by larger-scale studies, our results provide the first evidence for a role of p27 together with proliferation in predicting recurrence risk in a relatively small series of GIST. Further studies are needed to establish the relationship between these parameters and survival in these tumors.

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