ONCOLOGY REPORTS 16: 1029-1033, 2006

SPANDIDOS PUBLICATIONS

Significance of cyclin E and p27 expression in malignant ovarian germ cell tumors: Correlation with the cell proliferation activity and clinicopathologic features

SHIGETO KAWAUCHI[1], YOSHIAKI YAMAMOTO[1,2], KEN-ICHIRO UCHIDA[1,3], YASUYO CHOCHI[1], TOMOKO KONDO[1], ATSUNORI OGA[1] and KOHSUKE SASAKI[1]

Departments of [1]Pathology, [2]Urology and [3]Stomatology, Yamaguchi University School of Medicine, Yamaguchi 755-8505, Japan

Received April 10, 2006; Accepted July 21, 2006

Abstract. Although elevated cell proliferation activity is one of the most remarkable features known to characterize malignant ovarian germ cell tumors (MOGCTs), abnormalities in cell cycle regulation have yet to be thoroughly investigated in MOGCTs. Forty-two MOGCTs were immunohistochemically examined to determine their cyclin E and $p27^{Kip1}$ (p27) expression in correlation with their cell proliferation activity and clinicopathological features. Cytosine methylation of p27 gene promoter CpG islands was estimated by methylation-specific PCR. The labeling index (LI) was calculated as a percentage of the positively stained tumor cells per total tumor cells counted. p27 LIs showed a significant inverse correlation with Ki-67 LIs in MOGCTs examined ($p<0.05$). The cyclin E LIs of yolk sac tumors were significantly higher than those of dysgerminomas ($p<0.01$). Two MOGCTs were methylation-positive (5%). Both p27 and cyclin E are thought to be involved in the cell cycle regulation of MOGCTs. p27 gene promoter methylation is rare in MOGCTs. An immunohistochemical evaluation of cyclin E may be a useful diagnostic modality for differentiating MOGCTs.

Introduction

The cell cycle is regulated by balanced interactions among cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs) (1-3). Because autonomous cell proliferation is one of the most important features that characterize neoplasia (4), it is thought to be important to analyze the alterations of such cell cycle regulators in cancer research.

So-called G1 cyclins, including D-type cyclins (D1, D2 and D3) and cyclin E, are primarily involved in the G1-S transition of the cell cycle through interactions with specific cyclin-dependent kinases (CDKs) (3,5). Cyclin E is a late G1 cyclin that is associated with the conquest of the restriction point in the G1 phase of the cell cycle (6) and, along with its catalytic subunit CDK2, is involved in the phosphorylation of RB protein and the subsequent activation of E2F family transcription factors (3,5,6). E2F transcription factors promote the expression of various genes that are related to cell cycle progression and DNA synthesis (3,5). In several human malignancies, overexpression of cyclin E and cyclin E-related genes have been shown (8-10).

CDKIs associated with G1-S transition of the cell cycle are classified to two groups, the INK4 family and the CIP/KIP family (1-3). $p27^{KIP1}$ (p27) belongs to the CIP/KIP family and is a major regulator of cyclin E-CDK2 complex (3,5,6). The overexpression of p27 suppresses the kinase activity of cyclin E-CDK2 complex and the phosphorylation of RB protein, finally leading to G1 block in the cell cycle (3,5,6). p27 also serves as a substrate for cyclin E-CDK2 complex, and subsequent phosphorylation of p27 initiates its ubiquitination and degradation (ubiquitin-proteasome pathway) (3). A reduction or loss of p27 expression has been shown to be a marker of an increased cell proliferation and a poor prognosis in malignancies of various anatomic sites, including the breast (8), colon (7), urinary bladder (10), stomach (11), lung (12) and the soft tissues (13).

Malignant ovarian germ cell tumors (MOGCTs), consisting approximately 3% of all ovarian cancers, are made up of heterogeneous groups including dysgerminoma, yolk sac tumor, embryonal carcinoma, immature teratoma, other rare subtypes and their mixed tumors (14). Although an increased cell proliferation activity is one of the most remarkable features that characterize MOGCTs, abnormalities in cell cycle regulation have yet to be investigated fully (15). In the present study, to elucidate the significance of cyclin E and p27 in MOGCTs, we examined and analyzed the expression in correlation with the cell proliferation activity and some of their

Correspondence to: Dr Shigeto Kawauchi, Department of Pathology, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: shig@yamaguchi-u.ac.jp

Key words: malignant ovarian germ cell tumor, cell proliferation, cyclin E, p27, promoter methylation

clinicopathologic features. We also examined the p27 gene promoter CpG island methylation, an epigenetic mechanism that suppresses p27 gene expression (16), in MOGCTs.

Materials and methods

Tumor samples and mitotic count. Forty-two MOGCTs, from patients surgically treated from 1971 to 2003, were procured from the surgical pathology files of the Yamaguchi University Hospital and other related hospitals. The tumors were histologically diagnosed according to the World Health Organization classification (14). They comprised 22 dysgerminomas, 15 yolk sac tumors, 2 embryonal carcinomas and 3 immature teratomas. The age of the patients ranged from 11 to 45 years (mean, 23 years). Necrosis was observed in 12 tumors.

All MOGCTs were fixed in 10% neutral-buffered formalin and the embedded in paraffin wax. For each tumor, all the hematoxylin and eosin (HE) stained sections were reviewed, and the most representative one or two HE sections and the corresponding tumor tissue blocks were selected. The mitotic index (MI) was determined by counting the number of mitoses in four separate sets of 10 high power fields (x400) (10HPF) and then selecting the highest count.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tumor tissue sections (4-μm thick) were dewaxed in xylene and rehydrated through graded concentrations of ethanol solution. The endogenous peroxidase activity was blocked by 0.03% H_2O_2 in methanol. The sections were subjected to antigen retrieval pretreatment with 0.01 M sodium citrate buffer, pH 6.0, using a microwave processor (H2800, Energy Beam Science, Washington DC, USA) for 15 min. After blocking for non-specific binding sites with 2% normal rabbit serum, the sections were then forwarded to overnight incubation at 4˚C with optimally diluted primary antibodies against p27 (clone 1B4, 1:200, Novocastra, Newcastle upon Tyne, UK), cyclin E (clone 13A3, 1:50, Novocastra), and Ki-67 (clone MIB 1, 1:100, Immunotech, Marseille, France). A subsequent reaction was based on the streptavidin-biotin complex/horseradish peroxidase method using a Histofine SAB-PO (M) immunohistochemical staining kit (Nichirei, Tokyo, Japan) according to the method described by the manufacturer. A positive reaction was visualized with 3,3'-diaminobenzidine and then was counterstained with hematoxylin.

For an evaluation of each immunostained tissue section, the section was initially screened at a low magnification (x40), and then 3-4 separate areas containing tumor cells with distinct nuclear staining were selected for evaluation. The labeling indices (LIs) for p27, cyclin E and Ki-67 were defined as the percentage of tumor cells with positively stained nuclei per total tumor cells counted at a high magnification (x400). In each MOGCT, at least 1500 tumor cells were counted. The lymphocytes and fibroblasts served as internal controls.

Methylation-specific PCR. Formalin-fixed paraffin-embedded MOGCT tissue sections (10-μm thick) were dewaxed, rehydrated and stained with methylgreen (CosmoBio, Tokyo, Japan). The tumor tissue specimens were microdissected using a 28-gauge needle and then treated by proteinase K digestion and standard phenol/chloroform DNA extraction.

Methylation-specific polymerase chain reaction (PCR) for p27 gene promoter CpG island was performed according to the previously described method (17,18). Sample DNA (1 μg) was incubated at 55˚C with 3 M sodium bisulfite solution for 16 h to convert unmethylated cytosines to uracils while leaving the 5-methyl-cytosines unchanged. Modified DNA was subjected to PCR with primers specific for methylated (p27M primers; forward, 5'-CGATTAGTTAATTTTTC GGCGGC-3' and reverse, 5'-GCCGAAACTAACGAACG CG-3') or unmethylated (p27U primers; forward, 5'-AGTTA TGTGATTAGTTAATTTTTTGGTGGT-3' and reverse, 5'-CCCCACCAAAACTAACAAACACA-3') sequences (18). Template DNA (1.5 μl) was amplified in a total volume of 25 μl containing 1X universal PCR buffer, 2.5 mM dNTP mixture, 0.125 μmol/l of primers for p27M or p27U sequence, and 0.1 U of AmpliTag Gold polymerase (Applied Bios stems). The amplification conditions consisted of an initial denaturation at 95˚C for 10 min, followed by 38 cycles of 1 min each at 95, 55 and 72˚C, and a final extension at 72˚C for 7 min. The PCR products were electrophoresed on 2.5% agarose gel.

Methylation was considered to be positive when a 109-bp fragment that was amplified by MSP with p27M primers was present in a sample. All samples were evaluated by the presence of a 120-bp unmethylated-specific fragment that was amplified with p27U primers, which served as internal control for quality of sodium bisulfite-treated DNA.

Statistical analysis. Correlations among cyclin E LI, p27 LI, Ki-67 LI and MI were analyzed by either the Spearman rank correlation test or the Fisher's exact test. Correlations between clinicopathological factors and cyclin E LI, p27 LI, Ki-67 LI or MI were analyzed by the Mann-Whitney U test. A multiple regression analysis with stepwise variable selection was used to estimate the relative importance of factors that influenced Ki-67 LI. A two-tailed p-value of <0.05 was considered significant.

Results

Immunohistochemistry. Immunohistochemically, p27 and cyclin E were expressed predominantly in the tumor cell nuclei. In both dysgerminomas and yolk sac tumors, p27 showed diffuse and distinct staining pattern (Fig. 1A). Cyclin E showed sporadic and scattered staining pattern. Intense nuclear staining for cyclin E was more frequently observed in yolk sac tumors (Fig. 1B) than in dysgerminomas. In immature teratomas, distinct staining for cyclin E was exclusively observed in the glandular epithelium (Fig. 1C).

Cyclin E LI, p27 LI and Ki-67 LI ranged from 8 to 61% (mean, 42%), 51-97% (mean, 78%) and 4-45% (mean, 21%), respectively. MI ranged from 1 /10HPF to 23 /10HPF (mean, 12 /10HPF). Ki-67 LI and MI showed a significant linear correlation ($p<0.01$). Ki-67 LI demonstrated significant linear and inverse correlations with cyclin E LI ($p<0.05$) and p27 LI ($p<0.05$), respectively. However, MI showed a statistically significant correlation with neither cyclin E LI nor p27 LI. When MOGCTs were classified into two groups using a p27 LI cut-off value of 70%, Ki-67 LIs of MOGCTs with p27 LI <70% were significantly higher than those of MOGCTs with p27 LI ≥70% ($p<0.05$; Fig. 2).

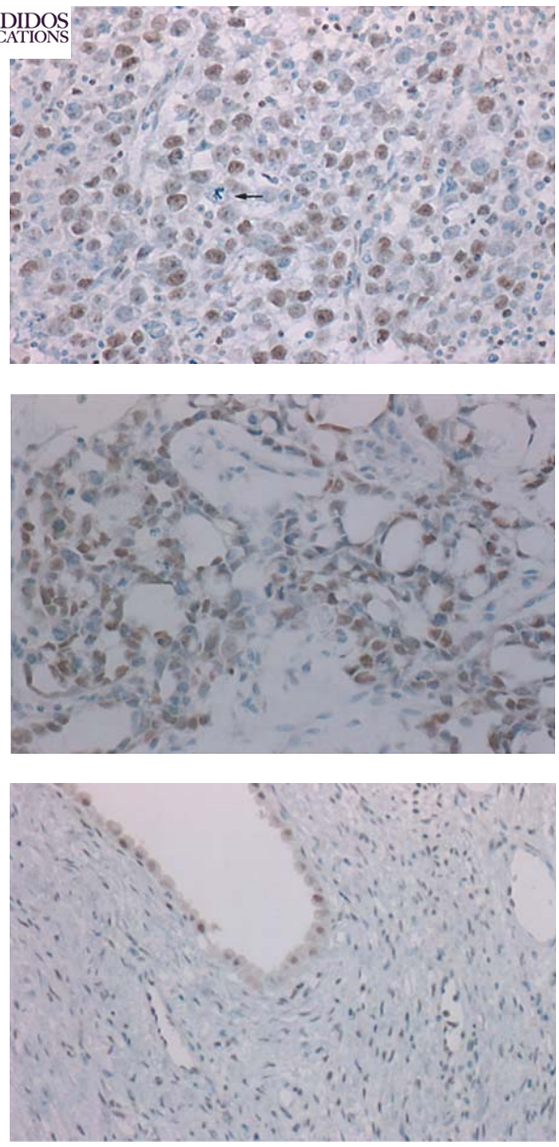

Figure 1. Immunohistochemical staining for p27 (A) and cyclin E (B and C). (A), p27 shows diffuse and distinct staining in the tumor cell nuclei. Mitotic nuclei are negative for p27 (small arrows) (dysgerminoma). (B), Intense nuclear staining for cyclin E is observed (yolk sac tumor). (C), Glandular cells are positive for cyclin E in the nuclei (immature teratoma).

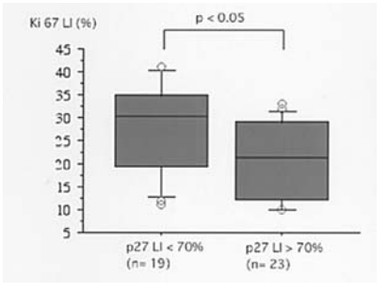

Figure 2. The difference in Ki-67 LIs between MOGCTs with p27 LI <70% and those with p27 LI ≥70%. The Ki-67 LIs in MOGCTs with p27 LIs <70% are significantly higher than those with p27 LIs ≥70% (p=0.037) (MOGCT, malignant ovarian germ cell tumor; LI, labeling index).

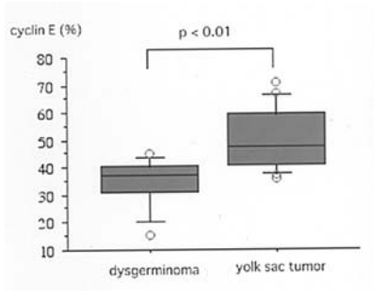

Figure 3. The difference in cyclin E LIs between dysgerminomas and yolk sac tumors. The cyclin E LIs of yolk sac tumors are significantly higher than those of dysgerminomas (p=0.008) (MOGCT, malignant ovarian germ cell tumor; LI, labeling index).

In a category data analysis, cyclin E LIs of yolk sac tumors were significantly higher than those of dysgerminomas ($p<0.01$, Table I, Fig. 3). p27 LIs of dysgerminomas tended to be higher than those of yolk sac tumors, but the difference was not statistically significant ($p=0.062$, Table I). Neither Ki-67 LIs nor MIs showed a statistically significant difference between dysgerminomas and yolk sac tumors (Table II). Cyclin E LI, p27 LI, Ki-67 LI and MI did not show any statistically significant differences when they were classified by categorized patient age or tumor necrosis (Tables I and II).

A multiple regression analysis with stepwise variable selection revealed that p27 LI was the most significant variable that influenced Ki-67 LI among the clinicopathologic features, cyclin E LI and p27 LI ($p<0.05$).

Methylation-specific PCR. Methylation-specific PCR for p27 gene promoter CpG island was successfully performed for 38 DNA samples extracted from 42 MOGCTs. Two (one dysgerminoma and one yolk sac tumor) of the 38 MOGCTs (5%) were methylation-positive for p27 gene promoter CpG island, while the remaining 36 MOGCTs were methylation-negative. Ki-67 LI and MI in methylation-positive MOGCTs tended to be higher than those in methylation-negative tumors.

Discussion

In our previous study, we reported that an occasional loss or a reduced expression of $p16^{INK4A}$ (p16) was associated with p16 gene promoter CpG island methylation and an increased cell proliferation in MOGCTs (19). Although p27 is a CDKI that has been advocated to be a useful marker to estimate the extent of cell proliferation activity and the prognosis of patients with a variety of malignancies, its significance has yet to be thoroughly investigated in MOGCTs. The present study was intended to analyze the expression of p27 and its competitive component, cyclin E, in correlation with the cell proliferation activity and some clinicopathological features in MOGCTs.

In the present study, we used two different markers to assess the cell proliferation activity, Ki-67 antigen (Ki-67 LI) and mitosis (MI). Ki-67 LI showed a significant correlation with p27 LI and cyclin E LI. However, MI did not show any such significant correlation. Because Ki-67 antigen is reported

Table I. Correlations of cyclin E and p27 with clinicopathological parameters in malignant ovarian germ cell tumors.

Clinicopathological parameters	n	Cyclin E LI[a] (%)	p-value[b]	p27 LI[a] (%)	p-value[b]
Age (years)					
<25	27	42.5±14.3	0.61	80.2±15.1	0.17
≥25	15	40.4±14.9		73.6±13.4	
Histological type					
Dysgerminoma	20	35.4±11.5	0.008	81.6±14.8	0.062
Yolk sac tumor	17	50.1±11.3		73.3±14.4	
Embryonal carcinoma	3	57.3		76.2	
Immature teratoma	2	20.1		73.3	
Necrosis					
Not	12	40.2±13.0	0.22	79.0±15.1	0.46
Any	30	46.2±16.2		75.3±13.6	

[a]LI, labeling index. Figures were expressed as mean or mean ± standard deviation. [b]Mann-Whitney U test.

Table II. Correlations of Ki-67 and mitosis with clinicopathological parameters in malignant ovarian germ cell tumors.

Clinicopathological parameters	n	Ki-67 LI[a] (%)	p-value[b]	MI[a] (/10HPF)	p-value[b]
Age (years)					
<25	27	23.9±9.8	0.91	12.2±3.9	0.32
≥25	15	23.3±8.5		13.3±3.4	
Histological type					
Dysgerminoma	20	22.1±8.9	0.087	13.1±3.9	0.39
Yolk sac tumor	17	27.6±9.9		12.2±3.8	
Embryonal carcinoma	3	24.0		14.0	
Immature teratoma	2	17.5		10.2	
Necrosis					
Not	30	23.9±9.5	0.97	12.6±3.8	0.92
Any	12	23.6±9.2		12.5±4.1	

[a]LI, labeling index; MI, mitotic index. Figures were expressed as mean or mean ± standard deviation. [b]Mann-Whitney U test.

to be expressed exclusively in proliferating cells from G1 to M phases, Ki-67 LI is likely to reflect the extent of cell proliferation more faithfully than MI (20). A multiple regression analysis with stepwise variable selection revealed p27 LI to be the only significant parameter that correlated with Ki-67 LI. Considering the result, p27 is thought to be more important for cell cycle regulation than cyclin E in MOGCTs.

Furthermore, in our study, cyclin E LIs of yolk sac tumors were significantly higher than those of dysgerminomas. No statistically significant differences between dysgerminomas and yolk sac tumors were detected in p27 LI, Ki-67 LI and MI. As a result, it is likely that cyclin E is involved in the differentiation of MOGCTs in addition to cell cycle regulation. In an immunohistochemical study of testicular germ cell

Datta *et al* reported that the extent of cyclin E n was associated with histological type of germ cell tumors (21). The results of Datta *et al* correlate with our findings and also support the hypothesis that cyclin E is involved in the tumor differentiation of germ cell tumors.

In the present study, a number of MOGCTs demonstrated a reduced p27 expression. p27 gene alterations that are responsible for the loss or reduction of p27 expression, such as silencing mutation and homozygous deletion, have been reported to be rare in human cancers (3,5). Recently, Nakatsuka *et al* reported frequent p27 gene promoter CpG island methylation and silencing of the p27 gene in hematological malignancies and lymphomas (22). Although we analyzed p27 gene promoter methylation in 38 MOGCTs, it was only rarely detected in the current study (5%). As a result, a reduced expression of p27 in most MOGCTs may be attributed to an activated post-transcriptional degradation mechanism, such as the ubiquitin-proteasome pathway.

A survival analysis with cyclin E LI and p27 LI failed to show a statistically significant improved survival in MOGCTs (data not shown). In the present study, the tumor samples mainly consisted of dysgerminomas and yolk sac tumors. Generally, dysgerminomas are much more sensitive to radiotherapy and chemotherapy than other MOGCTs. Furthermore, the tumor samples included in the present study all received various post-operative treatments. These factors might have adversely influence the analysis.

In summary, a reduced p27 expression and an increased cyclin E expression were associated with an increased cell proliferation in MOGCTs. The cyclin E expression was associated with the histological type of MOGCTs. An immunohistochemical evaluation of p27 is thought to be useful as a marker to estimate the cell proliferation activity and, presumably, the aggressiveness of MOGCTs. Furthermore, cyclin E may be useful as a diagnostic aid to differentiate MOGCTs. Further molecular studies and a case-controlled survival analysis will be necessary in the future to fully confirm the significance of our findings.

References

1. Sherr CJ: Cancer cell cycle. Science 274: 1672-1677, 1996.
2. Cordon-Cardo C: Mutations of cell cycle regulators. Am J Pathol 147: 545-560, 1995.
3. Sherr CJ: The pezcoller lecture: cancer cell cycles revised. Cancer Res 60: 3689-3695, 2000.
4. Rubin E and Farber JL: Neoplasia. In: Pathology. 2nd edition. Rubin E and Farber JL (eds). J.B. Lippincott, Philadelphia, pp143-198, 1994.
5. Pao P and Wilson T: G1 events and regulatio of cell proliferation. Science 246: 603-608, 1989.
6. Del Sai G, Loda M and Pagano M: Cell cycle and cancer: critical events at the G_1 restriction point. Crit Rev Oncol 7: 127-142, 1996.
7. Kitahara K, Yasui W, Kuniyasu H, *et al*: Concurrent amplification of cyclin E and CDK2 genes in colorectal carcinomas. Int J Cancer 64: 25-28, 1995.
8. Poter PL, Malone KE, Heagerty PJ, *et al*: Expression of cell-cycle regulators $p27^{kip1}$ and cyclin E, alone and in combination, correlates with survival in young breast cancer patients. Nat Med 3: 222-225, 1997.
9. Sgambato A, Doki Y, Schieren I and Weinstein IB: Effects of cyclin E overexpression on cell growth and response to transforming growth factor ß depend on cell context and p27kip1 expression. Cell Growth Differ 8: 393-405, 1997.
10. Del Pizzo JJ, Borkowski A, Jacobs SC and Kyprianou N: Loss of cell cycle regulators $p27^{kip1}$ and cyclin E in transitional cell carcinoma of the bladder correlates with tumor grade and patient survival. Am J Pathol 155: 1129-1136, 1999.
11. Mori M, Mimori K, Shiraishi T, *et al*: p27 expression in gastric carcinoma. Nat Med 3: 593-597, 1997.
12. Esposito V, Baldi A, Deluca A, *et al*: Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res 57: 3381-3385, 1997.
13. Kawauchi S, Goto Y, Liu XP, *et al*: Low expression of p27(Kip1), a cyclin-dependent kinase inhibitor, is a marker of poor prognosis in synovial sarcoma. Cancer 91: 1005-1012, 2001.
14. Nogales F, Talerman A, Kubik-Huch RA, Tavassoli FA and Devouassoux-Shisheboran M: Germ cell tumors. In: World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of the Breast and Female Genital Organs. Tavassoli FA and Devilee P (eds). IARC Press, Lyon, pp163-175, 2003.
15. Eyben FE: Chromosomes, genes and development of testicular germ cell tumors. Cancer Genet Cytogenet 151: 93-138, 2003.
16. Worm J, Bartkova J, Kirkin AF, *et al*: Aberrant p27Kip1 promoter methylation in malignant melanoma. Oncogene 19: 5111-5115, 2000.
17. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
18. Brakensiek K, Langer F, Kreipe H and Lehmann U: Absence of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ methylation in MDS and AML. Leukemia Res 29: 1357-1360, 2005.
19. Kawauchi S, Liu XP, Kawasaki K, *et al*: Significance of ß-catenin and pRB pathway components in malignant ovarian germ cell tumours: INK4A promoter CpG island methylation is associated with cell proliferation. J Pathol 204: 268-276, 2004.
20. Cattotetti G, Becker MHG, Key G, *et al*: Monoclonal antibodies against recombinant parts of the ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168: 357-363, 1992.
21. Datta MW, Renshaw AA, Dutta A, Hoffman MA and Loughlin KR: Evaluation of cyclin expression in testicular germ cell tumors: cyclin E correlates with tumor type, advanced clinical stage and pulmonary metastasis. Mod Pathol 13: 667-672, 2000.
22. Nakatsuka S, Liu A, Yao M, *et al*: Methylation of promoter region in p27 gene plays a role in the development of lymphoid malignancies. Int J Oncol 22: 561-568, 2003.