Oncogenic effect of Polo-like kinase 1 expression in human gastric carcinomas

YOUNG-JOO JANG[1], YONG SUNG KIM[2] and WOO HO KIM[3]

[1]Laboratory of Biochemistry, The School of Dentistry, Dankook University, 29 Anseo-Dong, Cheonan-Si, Chungnam 330-714; [2]Genome Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Oeun-Dong, Yusong-Gu, Daejeon 305-333; [3]Department of Pathology, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, Korea

Received April 10, 2006; Accepted June 5, 2006

Abstract. Polo-like kinase 1 (Plk1) is one of the serine/threonine kinases involved in various mitotic processes, such as centrosome maturation and chromosome segregation. Although Plk1 is increased in several tumor types, the effect of Plk1 in gastric cancer is not clear because of the limited patient numbers in previous studies. This study was performed to investigate the prognostic significance and the expression profiles of Plk1 in gastric carcinoma tissues from 280 Korean patients. The expression of Plk1 was analyzed by reverse transcriptional PCR and a tissue array using immunohistochemical staining method in gastric adenocarcinoma tissues. The oncogenic potential of Plk1 was analyzed by using Plk1-specific small interference RNA in gastric cancer cells. The message of Plk1 was increased in various gastric cancer cell types and in primary cancer specimens in comparison with normal tissue. The expression rate of Plk1 was 95% (268/280) in gastric carcinoma patients. Although Plk1 expression had no specific correlation in male or female patients, among the differentiation types of cancer, its expression was generally increased in gastric cancers. Plk1 expression was significantly associated with accumulation of proliferation-related genes and oncogenes, and reversely correlated with tumor suppressor genes. When Plk1 expression was blocked, cancer cell growth was inhibited and apoptotic phenotypes were detected. Overexpression of Plk1 was important in abnormal proliferation and showed oncogenic potential in gastric cancer. Plk1 might have potential as a tumor prognostic marker for gastric cancer.

Correspondence to: Dr Young-Joo Jang, Laboratory of Biochemistry, The School of Dentistry, Dankook University, 29 Anseo-Dong, Cheonan-Si, Chungnam 330-714, Korea
E-mail: yjjang@dku.edu

Key words: Polo-like kinase 1, gastric cancer, immunohistochemistry, siRNA, apoptosis

Introduction

Cancer is widely thought to be a genetic disease of various genetic abnormalities and uncontrolled cellular proliferation or programmed cell death. Genetic instability is one of the characteristic features of various cancers and is important in terms of carcinogenesis and tumor growth. Chromosomal abnormality is one form of genetic instability (1). Recent work has suggested that chromosomal instability can be induced by a spindle checkpoint defect (2). Frequent impairment of the mitotic checkpoint and a molecular analysis of the mitotic checkpoint genes, Mad1, Mad2, and Cdc20, has been reported and that disrupting these genes can lead to chromosomal instability (3-7). The centrosome plays an important role in maintaining chromosomal instability by bipolar spindle formation, and loss of chromosomal integrity is considered as one of the inducing factors during tumorigenesis. As well as chromosomal instability, centrosome abnormality is frequently observed in cancer cells (8,9).

The various events of the cell cycle, which is controlled by multiple signaling pathways, are considered to be important for causing cancer. A number of potential molecular targets for anticancer drug discovery have been identified in the cell cycle control mechanism (10,11). Prominent among them are cyclin-dependent kinases (CDKs) and the regulatory proteins, cyclins. Particularly, cyclins involved in the G1 phase of the cell cycle, such as cyclin D and cyclin E have been associated with human cancers, including lymphoma, breast, colorectal, prostate, and lung cancers (12-14).

One of the key regulators of the mitotic progression in mammalian cell cycle is the Polo-like kinase 1 (Plk1). The activity of Plk1 is increased in tissues and cells with a high mitotic index, including cancer cells (15-18). Plk1 has been reported to be involved in multiple processes including bipolar spindle formation, actin ring formation, centrosome maturation, and activation of the anaphase-promoting complex. Because of the roles of Plk1 in cellular proliferation, the level of Plk1 has been used as prognostic marker of several cancer types. The effect of Plk1 on centrosome abnormality has been studied. Lane and Nigg (19) found that injection of Plk1 antibody impair the ability

of cells to divide in both immortalized and non-immortalized human cells. The centrosomes in antibody-injected cells were found to be drastically reduced in size and abnormal distribution of bipolar spindle monoastral arrays were detected. Abnormal centrosomal phenotypes by uncontrolled Plk1 in cancer cells leads to chromosomal instability, causing aneuploidy, which may induce the changes of tumor related genes (20). Therefore, it is important to study the Plk1 funtions in tumorigenesis.

Gastric cancer is the most common cause of cancer death worldwide (21). This cancer is particularly prevalent in East Asian area, Korea and Japan, and is the leading cause of cancer death in these regions (22). Some report showed that aneuploidy and chromosomal aberrations were observed in gastric and liver cancers at the same frequency as in various other cancers (23,24). We performed a detailed analysis of Plk1 expression in primary gastric cancer cell lines and tumor tissues from Korean patients in comparison with normal tissues. We show that Plk1 has potential as a tumor marker for gastric cancer through the analysis of Plk1 oncogenic potential.

Materials and methods

Patients and tissue specimens. Two hundreds and eighty primary gastric cancer specimens and matched normal tissues were obtained by surgical procedures at the Department of Surgery, Seoul National University School of Medicine. The specimens were used for the immunohistochemical analysis and prepared for formalin-fixed, paraffin-embedded blocks.

Cell culture, RNA extraction, and RT-PCR. All gastric cancer cell lines (SNU 001, 005, 016, 216, 484, 520, 601, 620, 638, 668, and 719) (25) were cultured in RPMI supplemented with 10% fetal bovine serum. The total cellular RNA was extracted from the cells by column purification (Qiagen). Reverse transcriptase PCR (RT-PCR) for Plk1 was performed by one-step RT-PCR methods (iNtRON Biotech, Korea) in a 20-μl volume each containing 500 nM primer (sense Plk1, 5'-CTGCCTGCATCCCCATCTTC-3' and antisense Plk1, 5'-AGGCCTTGAGACGGTTGCTG). The reverse transcriptase (RTase) reaction was performed at 45˚C for 30 min, and at 94˚C for 5 min to inactivate RTase. The amplification was performed in the following steps: 30 sec at 94˚C (for denaturation, 1 cycle), 30 sec at 52˚C (for annealing, 25 cycles), and 30 sec at 72˚C (for extension, 1 cycle). After final extension of 5 min at 72˚C, PCR products were detected on 1.5% agarose gel. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as a control using the following primers (sense GAPDH, 5'-GTCAACGGATTTGGTCGTATT-3' and antisense GAPDH, 5'-AGTCTTCTGGGTGGCAGTGAT-3').

Immunohistochemistry. Immunohistochemical staining and evaluation were performed as previous reported (26,27). Surgical specimens were obtained from 280 patients with gastric cancer who underwent surgical resection at Department of Surgery, Seoul National University College of Medicine. Core tissue samples were taken from paraffin-embedded gastric tumors, and arranged in a tissue array block using trephine apparatus (26). A tissue block containing normal gastric mucosa was used as a control. Immunohistochemistry was performed using the labeled streptavidin-biotin immunoperoxidase technique to determine the expression of Plk1. Fixed samples were mounted on silane-coated glass slides, deparaffinized, and incubated with mouse anti-Plk1 monoclonal antibody (Zymed), diluted 100-fold in Tris-buffered saline pH 7.6 (TBS) with 1% BSA. In sequence, all the slides were incubated with secondary biotinylated antibody and peroxidase-labeled streptavidin, and examined under a light microscope. A negative control was performed with sections incubated without the primary Plk1 antibody. Sections were also immunostained with the anti-p53 monoclonal antibody, anti-RB monoclonal antibody, and other antibodies for detection of oncogene and tumor suppressors. Woo Ho Kim determined the final scores of each specimen.

Statistical analysis. Associations between Plk1 expression and various other parameters including clinicopathological characteristics were evaluated using the χ^2 test. The Mann-Whitney U test was applied to analyze the significance of differences between means. Survival rates were calculated starting from the day of surgery. The Kaplan-Meier method was used for overall survival curves, and differences were analyzed using the log-rank test. Statistical significance was assumed with a $P<0.05$.

Silencing of Plk1 by vector based-siRNA expression in gastric carcinoma cells. For silencing of Plk1 in gastric cancer cells, SNU-638 and SNU-719, plasmid pRNA-CMV3.1/Hygro (GenScript, SD1232) was used for vector construction. The targeting sequence of human Plk1 (accession no. NM_005030) was 5'-GGGCGGCTTTGCCAAGTGCTT-3' (coding region 183-203). Oligo DNA fragment was introduced into the pRNA-CMV3.1/Hygro (pRNA-Plk1). The plasmid without target DNA fragment was used as a control. To deplete Plk1 in SNU-638 cells, cells were transfected with pRNA-Plk1 by using Lipofectamine 2000™ (Invitrogen). At 24 h of post-transfection, the medium was changed, and 100 μg/ml of hygromycin was added for selection of the transfection-positive cells. Hygromycin selection was conducted for 7-10 days.

Apoptotic analysis by selection of annexin V-positive cells. Cells were harvested by trypsinization, and washed with ice-cold PBS. For staining of cell surface annexin V, FITC labeled annexin V-antibody was treated in cells for 30 min. Propidium iodide was also treated to detect dead cells. After staining, FITC-positive cells were analyzed by FACScan™ (Beckton Dickinson).

Results

Messenger RNA level of Plk1 in gastric carcinoma cell lines. We examined the message level of Plk1 in 11 different gastric carcinoma cell lines (Fig. 1). These cell lines were established from pathologically proven gastric carcinomas from Korean patients (25). Reverse transcriptase PCR was performed with total RNA isolated from 11 cancer cell lines (Fig. 1, lanes 1-11). The mRNA levels of Plk1 were also evaluated in total RNA from normal gastric tissue (Fig. 1, lane 12).

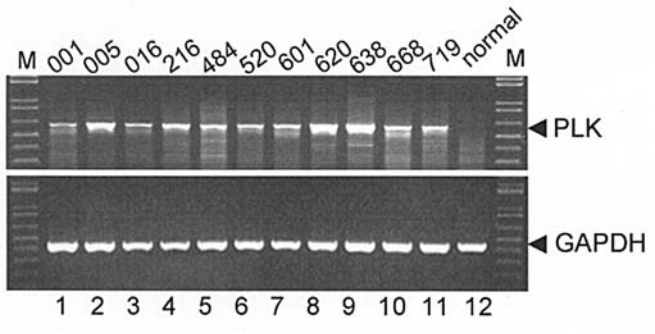

Figure 1. Representative profiles of the Plk1 message in various gastric cancer cell lines. RT-PCR products were obtained by agarose gel electrophoresis. GAPDH was used as an internal standard. Lanes 1-11, PCR products from total RNAs of the Korean gastric cancer cell lines (SNU-001, SNU-005, SNU-016, SNU-216, SNU-484, SNU-520, SNU-601, SNU-620, SNU-638, SNU-668, and SNU-719); Lane 12, PCR product from total RNA of normal tissue.

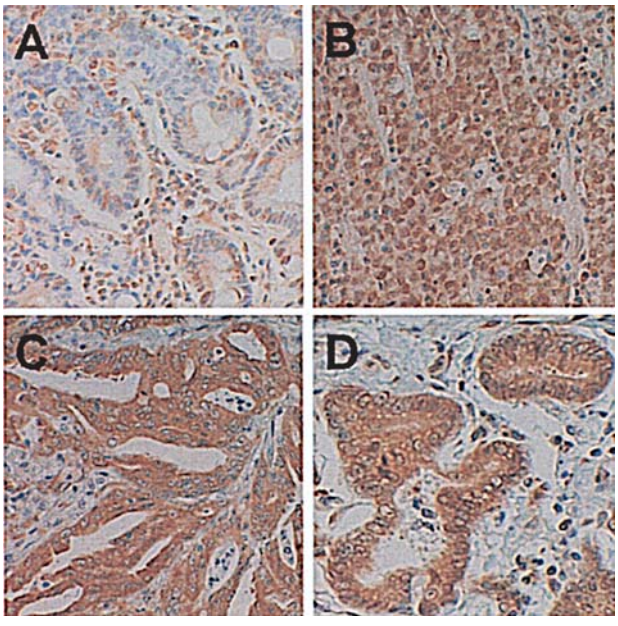

Figure 2. Immunohistochemical staining for Plk1 in gastric carcinoma tissues. (A) Staining of Plk1 in normal tissue. (B-D) Overexpression of Plk1 in gastric carcinoma tissues. Expression of Plk1 was observed mainly in the cytoplasm of cancer cells.

The expression level of Plk1 increased in all the carcinoma cell lines, whereas Plk1 was barely detectable in total RNA from normal tissue. These results were coincident to the cases of colorectal tumor, prostate cancer, and other cancer types (28).

In addition to the level of mRNA, we examined Plk1 protein levels in tumor specimens by immunohistological analysis. Although cells in normal gastric tissue showed weakly positive staining (Fig. 2A), 268 of 280 (95%) gastric carcinoma tissues showed strong intensity of Plk1 expression (Fig. 2B-D). Gastric cancer specimens were frequently strong or moderately positive according to the clinicopathological finding. Although there were 12 Plk1-negative cases (4.2%, 12 of 280 cases), we did not focus on them in detail in this study, but used these cases for calculating the P-value.

Table I. The correlation between Plk1 expression and the clinicopathological features in 280 gastric cancer patients.

	Plk1		
	Loss	Expression	P-value
Age (years)	56.92±11.5	54.21±13.2	0.486
Sex			0.034
Male	5	189	
Female	7	79	
Lauren classification			0.066
Intestinal	2	103	
Diffuse	10	133	
Mixed	0	32	
AGC vs. EGC			0.418
AGC	10	195	
EGC	2	73	
Depth			0.140
M	2	34	
Sm	0	39	
Pm	0	27	
Ss	9	112	
Se	1	56	
WHO classification			0.622
Well differentiated	0	26	
Moderately differentiated	2	74	
Undifferentiated	7	112	
Mucinous	1	14	
Signet ring	2	42	

Plk1 protein expression in gastric cancer tissue. Plk1 was positively stained in most of the cancer tissues by anti-Plk1 monoclonal antibody (Table I). The expression of Plk1 protein was detected in the nuclei as well as cytoplasm. Although the normal gastric foveolar epithelium is also positively stained, the cancer tissues were stained stronger and more homogenously. Significant relationships were not found between Plk1 expression and clinicopathological characteristics such as age, histological differentiation, and tumor location (Table I). Plk1 expression rate was slightly higher in men than in women (97.4% vs. 91.8%) and was higher in well differentiated than in poorly differentiated type (100% vs. 94.1%). Moreover, Plk1 expression showed a marginal statistical association with the Lauren classification as the relatively low rate (Table I, P=0.066), and the rate of mixed-type of tumor were higher than that of intestinal-type (100% vs. 98.1%) or diffuse type (100% vs. 93.0%). However, Plk1 expression increased without any significant correlations with the

Table II. Plk1 expression in 280 gastric cancer patients correlated to oncogenes and tumor suppressors.

Genes	Plk1		P-value
	Loss	Expression	
Cox-2			
-	5	25	0.001
+	7	227	
DCC			
-	8	50	0.000
+	4	202	
DNMT1			
-	4	2	0.000
+	8	262	
RB			
-	1	9	0.340
+	10	247	
p53			
-	10	162	0.120
+	2	97	
p63			
-	9	253	0.002
+	2	6	
Bcl-2			
-	11	227	0.500
+	1	32	
MUC2			
-	5	187	0.016
+	7	67	

clinicopathological characteristics in total cases, suggesting that Plk1 is one of the general marker proteins in gastric cancer.

Correlation between Plk1 and cancer-related gene expressions in gastric cancer tissues. Expression of Plk1 was significantly associated with the accumulation of proliferation-related genes and oncogenes. The elevated expression of DCC (deleted-in-colon-cancer) was observed in 206 cases (78%), and the expression of Cox-2 (cyclooxygenase-2) increased in 234 (88.6%) of 264 gastric cancers as indicated (Table II). In 202 of 264 gastric cancers, Plk1 and DCC were highly expressed simultaneously (Table II, P=0.00), while both Plk1 and Cox-2 were expressed in 227 cases of 264 specimens (P=0.01). The tumor suppressor such as p63 was correlated reversely with Plk1 expression. The expression of p63 protein was not detected in 262 of 270 Plk1-positive cancer tissues, whereas only 6 cases showed the expression of both Plk1 and p63 (P=0.02, Table II). The expression of p53, which is one of the

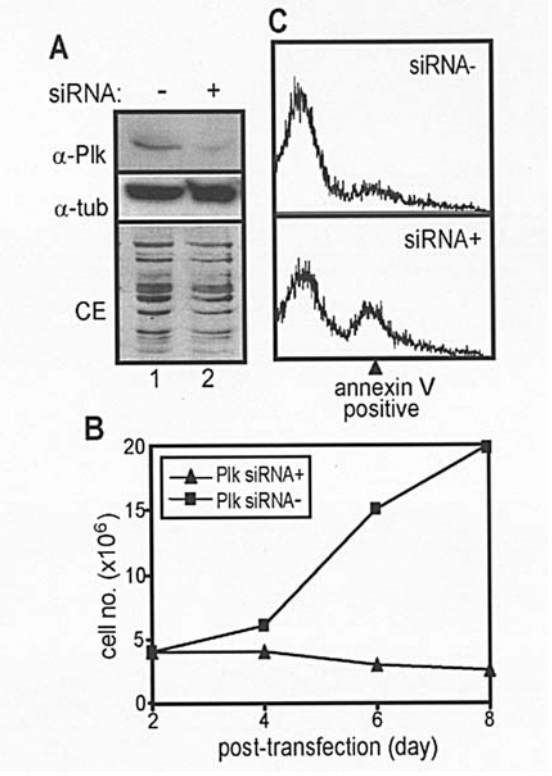

Figure 3. Growth inhibition of gastric cancer cells by Plk1 silencing using vector-based siRNA targeting Plk1. (A) SNU-638 cells were transfected with pRNA-CMV3.1/Hygro-Plk1 (siRNA$^+$). After transfection, cells were harvested, and cell extracts (CE) were subjected for Western blotting using the antibodies as indicated (Plk1 and tubulin). As a negative control, pRNA-CMV3.1/Hygro vector was transfected only (siRNA$^-$). (B) Growth inhibition by Plk1-silencing, cell proliferation was analyzed by counting the total cell. (C) Apoptotic phenotype in Plk1-silencing cancer cells by staining with FITC-labeled anti-annexin V antibody as described in Materials and methods.

representative tumor suppressors was reversely correlated with the expression of Plk1 (P=0.120, Table II). On the contrary, the expression of retinoblastoma (RB) protein was weakly correlated with Plk1 expression. These data suggested that Plk1 is one of the proliferation-related genes and may have an oncogenic potential like other cancer-related genes, such as DCC and Cox-2. Other representative expression profiles of Plk1 and cancer-related genes are shown in Table II.

Plk1 depletion inhibits cell proliferation and induces apoptosis in gastric cancer cells. To inhibit the expression of endogenous Plk1 in one of the cancer cell lines, SNU-638, which was established from a Korean gastric carcinoma patient (25), we used the vector-based siRNA technology developed recently (29). The targeting sequences of human Plk1 originated from the coding region 183-203 (16). The vector pRNA-Plk1 containing the targeting sequences was transfected into SNU-638 cells, and cells were cultured for 48 h after transfection. Cell extract was prepared and endogenous Plk1 amount was analyzed by Western blotting. Plk1 expression was efficiently depleted by this siRNA construct (Fig. 3A, upper panel, lane 2), whereas the level of tubulin was not changed by treatment of Plk1 siRNA construct in the same amounts of cell extract (Fig. 3A, middle panel, lanes 1 and 2).

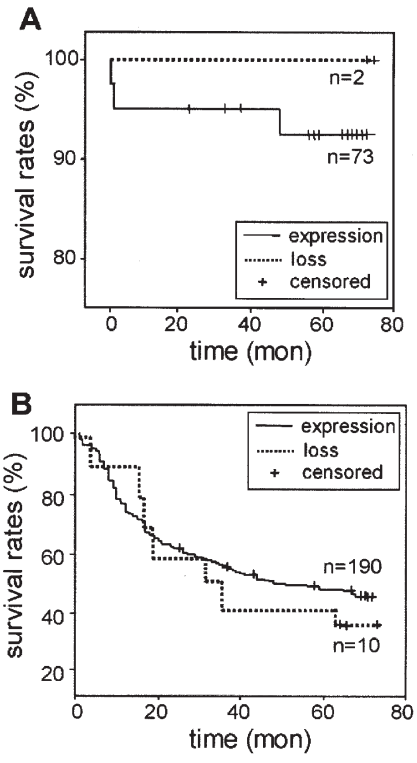

Figure 4. Survival distribution of Plk1-negative and Plk1-positive tumor patients in early gastric cancer (A) and the accurate gastric cancer stage (B).

We determined whether endogenous Plk1 depletion influences the cell growth of SNU-638 cells. The proliferation rate of cells transfected with a control vector, pRNA-CMV3.1/Hygro was not changed (Fig. 3B, siRNA$^-$), whereas the growth rate of cells expressing Plk1-targeting siRNA decreased (Fig. 3B, siRNA$^+$). Moreover, merely 10-20% of Plk1-depleted cells were normally attached on the culture dishes after more than 4 days of transfection. To analyze their apoptotic phenotype, cells were immunostained with FITC labeled anti-annexin V. Propidium iodide (PI) was treated for detection of necrotic cells. As expected, PI signal was only slightly detected, indicating that the necrotic cell lysis was not induced by Plk1 silencing (data not shown). Otherwise, Plk1-depleted cells were in apoptotic progression, and were annexin V-positive in FACScan analysis (Fig. 3C, lower panel). The Plk1 depletion also inhibited the proliferation rate of SNU-709 established from a gastric carcinoma patient (25) (data not shown), suggested that the depletion of Plk1 in the gastric cancer cell is sufficient to cause apoptosis.

Discussion

Plk1 is one of the important factors for cell proliferation. In mitosis, Plk1 regulates the spindle formation and the centrosome maturation, which are key mechanisms for progressing mitotic cell division. Abnormal ploidy is a common feature of cancer cells. In epithelial tumors, triploid and tetraploid DNA contents are detected frequently. Therefore, cancer cells contain a variety of gross chromosomal rearrangements, such as amplifications, deletions, or translocations (30). Centrosomes form the microtubule-organizing centre in the cells, and determine the accuracy of chromosomal segregation. Centrosomes are also required for the normal completion of cytokinesis (31). Because Plk1 is a key regulator of the centrosome function in mitosis, the abnormal level of Plk1 expression or activity may cause various defects in the cell cycle. Overexpression of Plk1 in murine fibroblasts NIH3T3 leads to the appearance of large cells with multiple or fragmented nuclei, which display a transformed phenotype in culture, and induces tumors when injected into nude mice (32). In tumor diagnosis, the increment of proliferation rate is an important feature for tumor progression, and most of the approaches have been adopted for its assessment, e.g. the proliferating cell nuclear antigen-labeling index (PCNA-LI). Because Plk1 level is also generally increased in rapidly proliferating cells (17), it could be a new index for estimating cancer progression. Extensive studies have shown that Plk1 expression is elevated in non-small cell lung cancer, melanomas, head and neck cancer, esophageal cancer, breast cancer, gliomas, and thyroid cancer. Based on these reports, Plk1 expression has been considered as a new prognostic marker for many types of malignancies, and proposed as a potential target for cancer therapy. Tokumitsu *et al* (33) reported that the message level of Plk1 was increased in gastric cancer. Because their approach was mainly to analyze the mRNA level, and the total cases were only 75, the correlation between Plk1 and gastric cancer is not clear. Here, we showed the prognostic possibility of Plk1 in gastric cancer by studying 280 cases of Korean cancer patients. Indeed, Plk1 was overexpressed in >95% of our cases. We also observed the loss of Plk1 gastric cancer-type in this experiment (4.28%, 12/280 cases). Because Plk1 is an essential gene for cell proliferation, it is difficult to explain the Plk1-loss type of cancer. However, Kaplan-Meier analysis, investigating the survival rate and Plk1 expression, indicated that high Plk1 expression can be correlated with poor survival in early gastric cancer as expected (Fig. 4A), although the significant correlation between the survival rate and Plk1 expression was diminished for the accurate cancer stage (Fig. 4B). Previously, several groups reported that DCC is one of the prognostic markers of colorectal cancer and has been used as a target for tailored chemotherapy in variable cancers (34,35). Additionally, the expression of Cox-2 is linked to angiogenesis, tumor cell growth, metastasis, and local immunosuppression in cancer progression (36), and has clinicopathological and prognostic significance in several types of cancer (37). Because Plk1 has an oncogenic feature, it was predictable that expression of Plk1 is correlated with expression of the tumor marker genes, such as DCC, Cox-2, DNMT-1 (DNA methyltransferase 1) (38,39), and MUC1 (mucin-1) (40) (Table II). Moreover, the expression of Plk1 was reversely associated with immunohistochemical state of tumor suppressors, p63 and p53, and was associated with the level of several oncogenes such as retinoblastoma protein. Takahashi *et al* (3) reported that Plk1 expression was associated with p53 accumulation in colorectal cancers. Although further study should be performed to explain the conflict between colorectal and gastric cancer, our data suggest that the functions of p53 are possibly different in various types of cancer. In spite of this conflict, the data

suggest that Plk1 expression in tumor was associated with the expression of proliferation-related genes or oncogenes, and that the combined evaluation of molecular biological markers may have advantages. In addition to overexpression of Plk1 in cancer, the growth inhibition and apoptosis induction by Plk1 depletion in gastric cancer cells suggested that Plk1 expression is important for oncogenic effect in tumor cells. In this report, we showed that the progression to primary gastric cancer is associated with the increasing expression levels of Plk1. According to the recent finding of a novel Plk1-specific inhibitor (41), the regulation of Plk1 activity in tumors by chemotherapeutic agents or gene therapy has been considered to be a valuable approach in cancer therapy.

Acknowledgements

This work was supported by R01-2004-000-10399-0 (2005) from Korea Science and Engineering Foundation, and from 21C Frontier Functional Human Genome Project (FG05-22-01) of the Korean Ministry of Science and Technology.

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