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Mutation and expression of the $p27^{KIP1}$ and $p57^{KIP2}$ genes in human gastric cancer

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Abbreviations: CDKI, cyclin-dependent kinase inhibitors; PCR-SSCP, polymerase chain reaction-single strand conformational polymorphism; DTT, dithiothreitol

Abstract

Cyclin-dependent kinase inhibitors (CDKI) are negative regulators of cell cycle progression by binding the cyclin-CDK complex and inhibiting the CDK activity. Genetic alteration in the CDKI genes has been implicated for carcinogenesis. To test the genetic alteration in the p27 and p57 genes, KIP family CDKI genes, 30 gastric tumor-normal pairs and 8 gastric cancer cell lines were analyzed for mutations by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP). No mutation was detected in these genes although length polymorphisms in the proline-alanine repeat of the p57 gene were detected. When the p27 and p57 mRNAs were analyzed in gastric cancer cell lines by RT-PCR, the p27 mRNA was expressed considerably high in tumor cells but expression of the p57 mRNA was much low in gastric cancer cell lines compared to that of normal cells. The result suggests that inactivation of gene expression rather than mutations in the p57 gene accounts possibly for the involvement of this gene in tumorigenesis of gastric cancer. However, expression of the p27 gene seems to be essential for cell survival.

Keywords: CDKI, PCR-SSCP, p27, p57, gastric cancer

Introduction

Cellular proliferation involves an orderly progression through the cell cycle that is controlled by protein complexes of cyclins and CDKs (Hunter *et al*., 1994; Cordoncardo *et al*., 1995; MacLachlan *et al*., 1995). The CDK subunits phosphorylate cell cycle-regulatory proteins, such as the retinoblastoma protein, to release cells from G1 arrest. The CDKIs are activated by wide variety of signals outside for growth arrest (at G1) and bind the cyclin D-CDK or cyclin E-CDK complexes to inactivate the catalytic domain of CDK. Mutations in the CDKI genes result in uncontrolled proliferation typical of neoplasia.

CDKIs are classified into two families, the CIP/KIP family and the INK4 family, based on primary sequence comparisons (Guan *et al*., 1994; Hannon *et al*., 1994; Cordon, 1995). Two families have distinct modes of actions (Hannon *et al*., 1994), as well as distinct structural characteristics. The INK4 proteins, $p16^{INK4a}$ (Serrano *et al*., 1993), $p15^{INK4b}$ (Hannon *et al*., 1994), $p18^{INK4c}$ (Guan *et al*., 1994; Hirai *et al*., 1995), and $p19^{INK4d}$ (Chan *et al*., 1995) are highly related to each other in containing the ankyrin-type repeats. They are reported to be frequently mutated in a wide variety of cancers. The CIP/KIP family CDKIs, $p21^{CIP1/WAF1}$ (Xiong *et al*., 1993), $p27^{KIP1}$ (Polyak *et al*., 1994; Toyoshima *et al*., 1994), and $p57^{KIP2}$ (Lee *et al*., 1995; Matsuoka *et al*., 1995) share common sequence motifs that mediate interaction between the CDKI and cyclin-CDK complexes (Koufos *et al*., 1989; Hunter *et al*., 1994; Hirai *et al*., 1995). $p21^{CIP1/WAF1}$ was the first CDKI to be identified, and it is induced by p53, transforming growth factor-β (TGF-β), differentiation, and cellular senescence (Lee *et al*., 1995). $p27^{KIP1}$ is appears to be involved in G1 arrest induction by cell to cell contact, cyclin AMP-inducing agents, and repamycin (Koufos *et al*., 1989). $p57^{KIP2}$ is unique in that it has a prolin-rich region and an acidic domain. The p27 protein has 42% amino acid homology with p21 and 47% similarity with the p57 protein in the N-terminal domain which mediates inhibition of CDK (Konstantin, 1996). In contrast to the ubiquitous expression of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, $p57^{KIP2}$ is expressed at high levels in specific embryonic and adult tissues (Hunter *et al*., 1994).

Three genes, p21, p27, and p57 have been investigated in different kinds of human tumors. Unlike the INK4 family, only few genetic alterations have been found. This suggests that mutational inactivation of these CDKIs is infrequent (Loh *et al*., 1992), but gene inactivation by alternative mechanisms seems to be the

general pathway. To elucidate whether CDKI genes are mutated in gastric cancer, 30 of gastric tumor-normal pairs and 9 gastric carcinoma cell lines were analyzed for structural integrity of p27 and p57 CDKI genes by PCR-SSCP. mRNA expression of these genes in 9 gastric cancer cell lines was tested by RT-PCR.

Materials and Methods

Tumor samples and cell lines

Tumor samples were obtained at the time of surgery from 30 patients with primary gastric carcinoma in Hallym University Hospital. The corresponding normal gastric tissues surrounding the tumors were also obtained to compare. The resulting tissues were frozen immediately in liquid nitrogen and stored at -70°C before analysis. Nine gastric cancer cell lines (SNU1, SNU5, SNU16, SNU484, SNU620, SNU638, SNU668, SNU719, KATO III) were obtained from KCLB (Korea Cell Lines Bank). Gastric cancer cells were cultured in RPMI 1640 (GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum (GIBCO-BRL) and maintained at 37°C and 5% CO_2.

DNA isolation

Tissue samples were digested at 50°C in digestion buffer containing 1% sodium dodecyl sulfate and proteinase K (0.1 mg/ml) overnight. DNA was extracted with phenol-chloroform and precipitated with cold ethanol at -20°C. DNA precipitate was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in TE buffer solution (10 mM Tris, 1 mM EDTA pH 8.0).

PCR-SSCP analysis

The location of PCR primer pairs for the amplification of the entire coding region of the human p27 and p57 genes and the size of PCR products generated by them are illustrated Figure 1. PCR reaction for the p27 gene was performed from 100 ng of genomic DNA in 20 μl of the reaction buffer containing 10 pmol of each of the primer, 0.1 mM of each of the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (Perkin Elmer). Three sets of primers used to amplify the exon 1, 2 and 3 of the human $p27^{KIP1}$ gene as described previously (Gombart *et al.*, 1995). The sequences of primers were summarized in Table 1. PCR conditions for each exon were as follows: p27 exon 1: 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C; p27 exon 2: 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C; p27 exon 3: 35 cycles of 1 min at 95°C, 1 min at 65°C, 1 min at 72°C, followed by 72°C for 10 min. Primer set 1, 4 and 5 regions of the p57 genes were amplified from 100 ng of genomic DNA in 20 μl of the reaction buffer containing 10 pmol each of the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (Perkin Elmer).

The five sets of primers were prepared to amplify the entire coding region of the human $p57^{KIP2}$. The location of primer sets and the size of PCR products generated by them are illustrated in Figure 1 and the sequences of primers were listed in Table 1. PCR condition for each set was as follows: p57 set I: 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C; p57 set IV: 35 cycles of 1 min at 95 °C, 1 min at 60°C, 1 min at 72°C; p57 set V: 35 cycles of 1 min at 95°C, 1 min at 58°C, 1 min at 72°C, followed by 72°C for 10 min. Set II and III regions of the p57 gene which contain CG rich sequences were amplified from 100 ng of genomic DNA in 20 μl of the reaction buffer (pH 8.5) containing 10 pmol of each the primer 0.125 mM of each of the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 1.25 U of pfu DNA polymerase (Stratagene). PCR condition for each set was as follows: 35 cycles of 1 min at 98°C, 1 min at 65°C, 1 min at 74°C, followed by 74°C for 10 min. PCR products were mixed with an equal volume of formamide loading dye (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue). Samples were denatured at 95°C for 5 min and resolved on 8% nondenaturing polyarylamde gels containing 10% glycerol. Resulting gels were visualized by silver staining according to the manufacture's instructions (Promega)

RNA isolation and RT-PCR

To analyze the RNA expression, total RNA was isolated from 2×10^6 cells by lysis in 0.2 ml of RNAzol B (Bio-Tecx Inc.). RNA was precipitated in 50% isopropanol,

Table 1. Sequences of primer sets for SSCP-PCR and RT-PCR

Gene		Sequence of forward primer	Sequence of reverse primer
p27	Set I	F: ATGTCAAACGTGCGAGTGTC	R: TCTGTAGTAGAACTCGGGCAA
	Set II	F: TTGCCCGAGTTCTACTACAGA	R: AGGGTCATTACCGTCGGTTGC
	Set III	F: CCCCTGCGCTTAGATTCTTCT	R: CGTTTGACGTCTTCTGAGGCC
p57	Set I	F: TCGCTGCCCGCGTTTGCGCA	R: CCGAGTCGCTGTCCACTTCGG
	Set II	F: CTTCCAGCAGGACATGCCGCT	R: TGGAGCCAGGACCGGGACT
	Set III	F: GCTGCCTAGTGTCCCGGTC	R: GATCAGAGGCCCGGACAGCTT
	Set IV	F: CGCAGATTTCTTCGCCAAGCG	R: AGGTGCGCTGTACTCACTTGG
	Set V	F: GTGAGCCAAGTGAGTACAGC	R: GGGACCAGTGTACCTTCTCG

extracted with chloroform, and collected by centrifugation (15 min at 12,000 × g). The RNA pellet was washed twice in 70% ethanol and dissolved in diethylpyrocarbonate-treated double-distilled water. The reverse transcriptase reactions were performed by incubation at 52°C for 20 min in a 20 μl reaction containing RNA (100 ng), reverse primers, 1 mM deoxynucleotide triphosphates (Boehringer Mannheim), reaction buffer (50 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$, provided by Takara Shuzo Co.) and AMV reverse transcriptase (Takara Shuzo Co.). cDNA was amplified using the p27 primer set I for p27 and the p57 primer set I for p57. The PCR reaction for p27 was performed as follows: 95°C for 8 min, 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, and followed by incubation at 72°C for 10 min. The PCR reaction for p57 was performed as follows: 95°C for 8 min, 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, and followed by incubation at 72°C for 10 min. PCR products were resolved on 2% agarose gels.

Results and Discussion

Mutation analysis of the p27 gene

Thirty gastric tumor-normal pairs and 8 gastric cancer cell lines were analyzed for mutations of the p27 genes by PCR-SSCP. Three sets of primers that cover the entire coding region of the p27 gene containing 3 exons were used (Figure 1) for PCR-SSCP. No shifted band was detected in all PCR-SSCP results obtained from 3 different sets of primers for gastric tumor-normal pairs (Figure 2) and 8 gastric cancer cell lines (Figure 3). The result indicates that neither mutation nor polymorphism exists in the coding region of the p27 gene in gastric cancer cells.

Expression of the p27 mRNAs in gastric cancer cells

The expression of the p27 mRNA was analyzed in 8 gastric cancer cell lines by RT-PCR. The expression level of the p27 mRNA was considerably high in all 8 cell lines when compared to that of normal fibroblast cells (Figure 4). This result indicates that the p27 gene has no mutation and is actively expressed even in cancer cells. Expression of p27 seems to be essential for cell survival. So an intact p27 protein should be prepared in cancer cells as in normal cells even though the p27 protein is growth-inhibitory. However, the result of the p27 knock-out mouse does not support this (Cordon *et al*., 1998). Deletion of the p27 gene did not produce abnormal cell growth or cancer formation. Presence of other CDKI may substitute the function of p27 in the p27 knock-out mouse. Similar substitution did

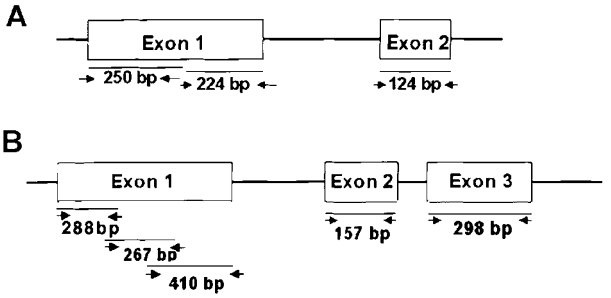

Figure 1. Location of PCR primers for PCR-SSCP in the p27^{KIP1} gene (A) and the p57^{KIP2} gene (B) and size of PCR product.

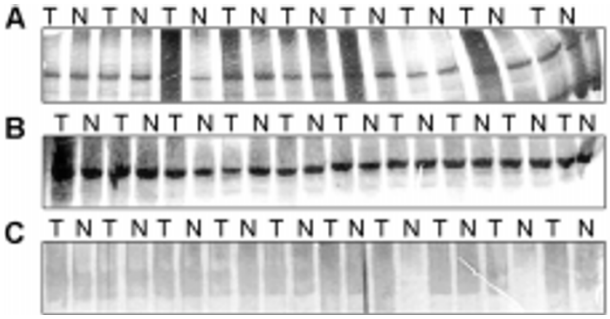

Figure 2. PCR-SSCP anslysis of the p27 gene for DNA from gastric tumor-normal pairs. DNA was amplified using primer set I (A), set II (B), and set III (C). N, normal; T, tumor.

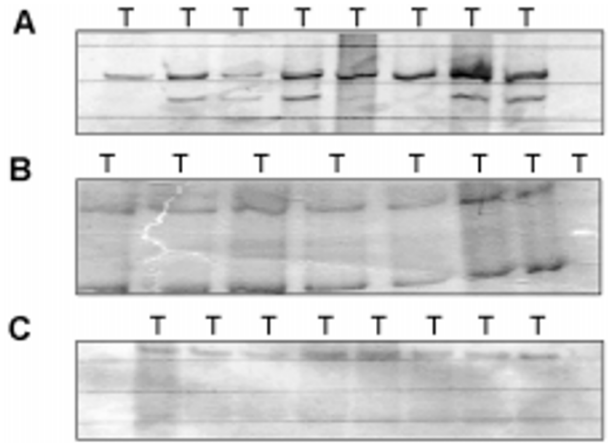

Figure 3. PCR-SSCP anslysis of the p27 gene for DNA from gastric cancer cell lines. DNA was amplified using primer set I (A), set II (B), and set III (C).

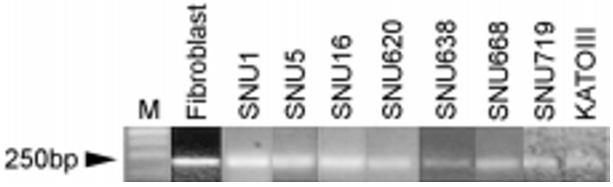

Figure 4. mRNA expression of the p27 gene in gastric cancer cell lines. mRNA expression of p27 was measured by RT-PCR using total RNA 100 ng. RT-PCR product was resolved in 2% agarose gel. Fibroblast was analyzed as a control.

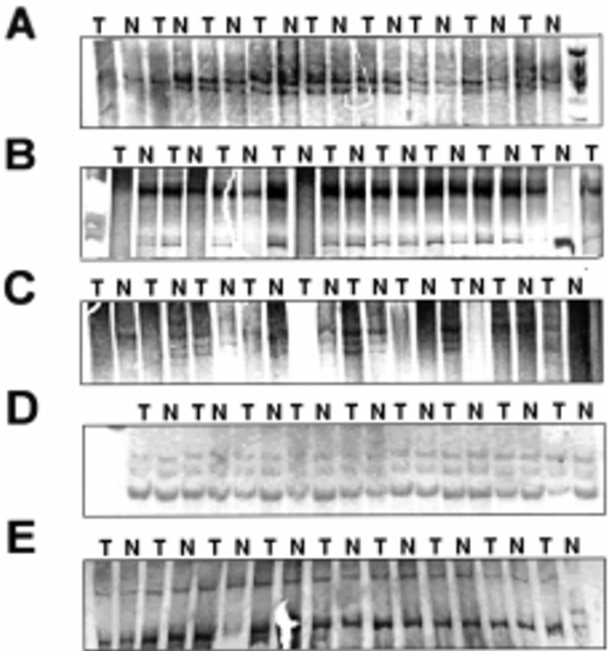

Figure 5. PCR-SSCP anslysis of the p57 gene for DNA from gastric tumor-normal pairs. DNA was amplified using primer set I (A), set II (B), set III (C), set IV (D), and set V (E), N, normal; T, tumor.

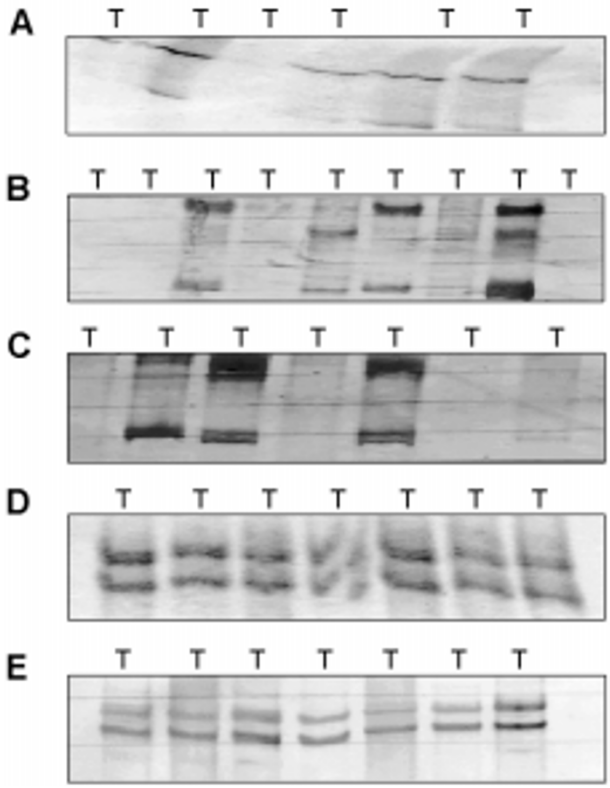

Figure 6. PCR-SSCP anslysis of the p57 gene for DNA from gastric cancer cell lines. DNA was amplified using primer set I (A), set II (B), set III (C), set IV (D), and set V (E).

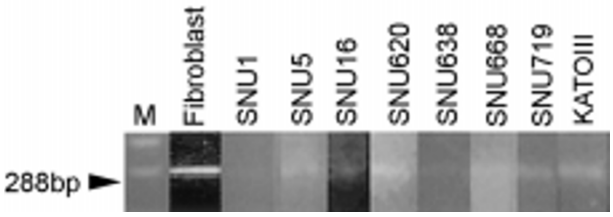

Figure 7. mRNA expression of the p57 gene in gastric cancer cell lines. mRNA expression of p57 was measured by RT-PCR using total RNA 100 ng. RT-PCR product was resolved in 2% agarose gel. Fibroblast was analyzed as a control.

not happen here in gastric cancer cells. Other KIP family CDKI genes, p21 (data not shown) and p57 (Figure 7), are inactivated in gastric cancer cells. Only p27 gene seems to be active in maintaining the minimal function for cell survival.

Mutation analysis of the p57 gene

To investigate the presence of mutations in the p57 gene, DNA from 30 gastric tumor-normal pairs and 8 gastric tumor cell lines were analyzed using PCR-SSCP. No mutation was detected in exon 1, exon 2, and exon 3 (Figure 5 and 6). However, in PCR-SSCP using primer set II and III, band shift was observed in both normal and tumor tissues (Figure 5 and 6). As previously reported (Matsuoka *et al.*, 1996), we also observed (data not shown) the length polymorphism in the proline-alanine repeat of exon 2, and it is very likely that the shifted bands were due to polymorphism and not to mutations. The result suggests that there is no mutation in the p57 gene in gastric cancer.

Expression of the p57 mRNAs in gastric cancer cells

RT-PCR was used to determine the expression of the p57 mRNAs in 8 gastric cancer cell lines. The expression level of the p57 mRNAs was very low in gastric cancer cell lines compared to that of normal human fibroblast (Figure 7). The result indicates that there is no mutation in the p57 gene but it is inactivated in gastric cancer cells. Inactivation of gene expression rather than mutations in the p57 genes appears to be the mechanism of cell growth in gastric cancer cells. The understanding of of p57 gene inactivation may provide closer insight to development of gastric neoplasia.

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