Methylation Analysis of Cyclin-Dependent Kinase Inhibitor Genes in Primary Gastrointestinal Lymphomas

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The CIP/KIP family of cyclin-dependent kinase inhibitors may act as tumor suppressors. To assess promoter hypermethylation as a potential underlying mechanism for loss of expression, methylation-specific polymerase chain reaction for p21 and p27 genes was performed in 13 gastric low-grade mucosa-associated lymphoid tissue (MALT) lymphomas, 13 gastric high-grade B-cell lymphomas, and 14 intestinal diffuse large B-cell lymphomas. p21 and p27 genes were unmethylated in normal Peyer's patch and tonsillar tissues. Promoter hypermethylation of p21 gene was detected only in some gastric low-grade MALT lymphomas (4/13, 31%). All gastric and intestinal high-grade lymphomas revealed unmethylated status of p21 gene. p27 gene was unmethylated in all cases of low- and high-grade gastrointestinal lymphomas. These results suggest that p21 promoter methylation is involved in some low-grade MALT lymphomagenesis in stomach and seems to be an early event in the gastric lymphomagenesis. And promoter methylation is not the underlying mechanism for loss of p27 protein expression in the malignant lymphomas of the stomach and intestine.

KEY WORDS: Lymphoma, Methylation, p21, p27.
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The control of mammalian cell cycle involves a family of proteins that bind to and inhibit the cyclin-dependent kinases (CDK; 1). Recent biochemical and genetic studies suggest that these CDK inhibitors may act as tumor suppressors (2). Based on the comparative studies of amino acid sequences and biochemical properties, two classes of CDK inhibitors, INK family and CIP/KIP family (3), have been identified (2). The INK family includes p16, p15, and p18 (2) and the CIP/KIP family includes p21, p27, and p57 (3).

p21 (WAF, Cip1) is a small protein that affects the function of most known cyclin/CDK complexes, blocking DNA replication and cell cycle progression into S phase (3). p21 is induced by p53, transforming growth factor β, differentiation, and cellular senescence (4). p21 seems to induce cell cycle arrest and apoptosis in actively proliferating cells and is an important factor regulating the cell cycle in proliferating cells (5). Overexpression of p21 results in the growth inhibition of colon cancer cells, brain tumor cells, and leukemia cells and can induce growth arrest and apoptosis of human carcinoma cell lines (1).

p27 protein has 42% of homology with p21 in its *N*-terminal region and binds to cyclin E/CDK2 and/or cyclin D/CDK4 complexes, thus blocking progression from G1 to S phase of the cell cycle (6). p27 is generally expressed at high level in quiescent cells, and constitutive overexpression of p27 in cultured cells causes inhibition of cell proliferation and cell cycle arrest in G1. The activity of p27 is up-regulated *in vitro* by a variety of extracellular antiproliferative signals, including transforming growth factor, cell–cell contact and growth factor depletion. Over a wide range of tumor types, decreased expression of p27 is a marker of disease progression and an adverse prognostic factor (7).

In my previous immunohistochemical studies on primary gastric lymphomas, all low-grade mucosa-associated lymphoid tissue (MALT) lymphomas were positive, but all high-grade B-cell lymphomas were negative for p27 protein (8). And p21 was rarely expressed in both low- and high-grade B-cell lymphomas of the stomach by immunohistochemistry (9). In contrast, p21 and p27 were frequently expressed in primary intestinal large B-cell lymphomas by immunohistochemistry, and p27-positive cases had a much higher percentage of patients sustaining a continuous complete remission state as compared with p27-negative cases, suggesting that p27 immunoreactivity may be associated with

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better clinical outcome in primary intestinal large B-cell lymphoma (10). The objective of the present study was to assess p21 and p27 promoter hypermethylation in primary gastrointestinal lymphomas as a potential underlying mechanism for their altered expressions.

MATERIALS AND METHODS

Tumor Samples and DNA Extraction

Paraffin-embedded tissue blocks from 40 cases of primary gastrointestinal lymphomas were retrieved from the Department of Pathology, Dankook University College of Medicine; the Department of Pathology, Yonsei University College of Medicine and the Department of Diagnostic Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine in Korea. In the stomach, the diagnosis of low-grade lymphoma of the MALT type was made based on the well-established histologic criteria described by Isaacson (11). Lymphomas predominantly composed of large transformed cells were diagnosed as high-grade B-cell lymphomas, irrespective of low-grade component. Thirteen were low-grade gastric lymphomas of the MALT type, and 13 were high-grade B-cell lymphomas. In the intestine, all 14 cases were diagnosed as diffuse large B-cell lymphoma according to the World Health Organization classification (12). No low-grade components were identified histologically. The B-cell nature of these tumors was confirmed by the immunohistochemical detection of the B- (CD20) and T-cell (CD3 or UCHL) markers using paraffin sections. Paraffin tissue blocks from three cases of non-neoplastic tonsillar tissues and one case of Peyer's patch were also retrieved as controls. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues as previously described.

Methylation-Specific Polymerase Chain Reaction

DNA methylation patterns in the CpG islands of the p21 and p27 genes were determined by methylation-specific polymerase chain reaction (13). Methylation-specific polymerase chain reaction distinguishes unmethylated from methylated alleles based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated cytosine to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA (14). Sodium bisulfite modification was performed using a CpGenome DNA Modification kit (Intergen, Oxford, UK) according to the manufacturer's protocol. Briefly, DNA was denatured by NaOH (final concentration, 0.2 M) for 15 minutes at 37° C. Sodium bisulfite solution at pH 5, freshly prepared, was added (550 μL), and incubated at 50° C for 20 hours. The modified DNA was treated with NaOH (final concentration, 0.3 M) for 5 minutes at room temperature, followed by ethanol precipitation, and was resuspended in TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA; 14). The primer sequences for p21 and p27 were as follows: 5'-TTG GGC GCG GAT TCG TC-3' (sense) and 5'-CTA AAC CGC CGA CCC GA-3' (antisense) for the p21 methylated reaction; 5'-TTA GTT TTT TGT GGA GTT G-3' (sense) and 5'-CTC AAC TCT AAA CCA CCA A-3' (antisense) for the p21 unmethylated reaction; 5'-AAG AGG CGA GTT AGC GT-3' (sense) and 5'-AAA ACG CCG CCG AAC GA-3' (antisense) for the p27 methylated reaction; and 5'-ATG GAA GAG GTG AGT TAG T-3' (sense) and 5'-AAA ACC CCA ATT AAA AAC A-3' (antisense) for the p27 unmethylated reaction (15). PCR was carried out in a 10-μL volume containing PCR buffer (10 mM Tris pH 8.3, 50 mM KCl), 1.5–2.0 mM $MgCl_2$, 250 μM dNTPs, 0.5 μM of each primer, 0.5 U *Taq* DNA polymerase, and 40 ng of bisulfite-modified DNA. Amplification was performed on a thermal cycler (Perkin Elmer, Norwalk, CT) and comprised initial denaturing at 95° C for 5 minutes followed by 35 cycles of denaturing at 95° C for 45 seconds, annealing for 45 seconds at 62° C (for p21 methylated and unmethylated reactions) or 66° C (for p27 methylated and unmethylated reactions), and extension for 1 minute at 72° C, followed by a final extension for 4 minutes at 72° C. PCR products were electrophoresed on 3% agarose gels and were visualized by ethidium bromide (14).

RESULTS

In the present study, promoter hypermethylation of p21 gene was detected in 4 of 13 low-grade MALT lymphomas of the stomach. No promoter hypermethylation of p21 gene was detected in all cases of high-grade lymphomas of the stomach and intestine (Fig. 1). p27 genes were unmethylated in all low- and high-grade lymphomas of the gastrointestinal tract (Fig. 2). One case of Peyer's patch and three cases of tonsillar tissues also revealed unmethylated status of p21 and p27 genes. The representative cases were shown in Figures 1 and 2.

DISCUSSION

The genes of CIP/KIP family CDK inhibitors have been investigated in many different kinds of human tumors and, unlike the case with the INK family, only a few genetic alterations have been found (4). With regard to lymphoma, p21 alterations have been analyzed only in a few studies. A role for deletion and loss of expression of p21 in aggressive variants of mantle cell lymphomas has been pro-

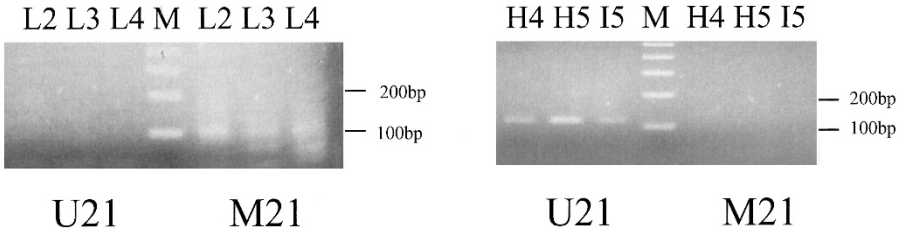

FIGURE 1. Methylation-specific PCR of p21 genes. Promoter hypermethylation of p21 gene was detected in 4 of 13 low-grade MALT lymphomas of the stomach. Representative cases showing methylated p21 genes in L2, L3, and L4 (**left**). p21 was unmethylated in all high-grade B-cell lymphomas of the stomach and intestine. Representative cases showing unmethylated p21 genes in H4, H5, and I5 (**right**). L2, L3, L4 = low-grade MALT lymphomas of the stomach; H4, H5 = high-grade B-cell lymphomas of the stomach; I5 = diffuse large B-cell lymphoma of intestine; M = molecular weight marker; U21 = unmethylated primer for p21; M21 = methylated primer for p21.

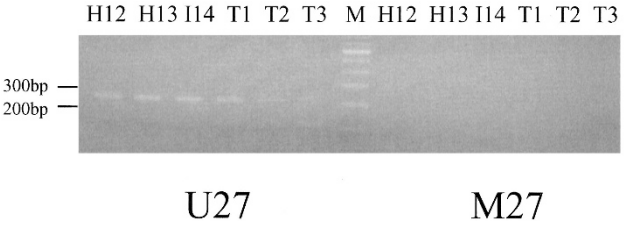

FIGURE 2. Methylation-specific PCR of p27 genes. All low- and high-grade B-cell lymphomas of the stomach and intestine showed unmethylated status of p27 gene. Representative cases showing unmethylated p27 genes in H12, H13, I14, T1, T2, and T3. H12, H13 = high-grade B-cell lymphomas of the stomach; I14 = diffuse large B-cell lymphoma of intestine; T1, T2, and T3 = nonneoplastic tonsils; M = molecular weight marker; U27 = unmethylated primer for p27; M27 = methylated primer for p27.

posed (16), but other investigators failed to identify mutations of this gene in a large series of human malignancies including hematologic tumors (17). Specific alterations of p27 gene, including mutations and homozygous deletion, are exceedingly rare in human cancers, including systemic non-Hodgkin's lymphomas (7). The results so far suggest that the mutational inactivation of these CIP/KIP family CDK inhibitors is infrequent but that gene inactivation by alternative mechanisms seems to be the general pathway (4).

Two known mechanisms, gene inactivation by methylation in the promoter region and change to an inactive chromatin by histone deacetylation, seem to be the best candidates for inactivation of CIP/KIP family CDK inhibitor genes, because these two mechanisms are the most recently and frequently reported mechanisms for inactivation of specific genes (4). However, there have been limited data regarding the methylation status of p21 and p27 genes in malignant tumor, especially in the malignant lymphomas.

In a study of primary central nervous system lymphomas, p21 expression was relatively low in many tumors and promoter hypermethylation was not detected, even in those without p21 expression (15). Therefore, it was suggested that the p21 expression might be regulated at the transcriptional level, although the significance of p21 in the development of lymphomas is still unclear (15). In the present study, p21 methylation was identified in 4 of 13 cases of low-grade MALT lymphomas, but not in high-grade B-cell lymphomas of the stomach. Therefore, it can be suggested that p21 promoter hypermethylation may be involved in gastric lymphomagenesis as an early event. In contrast, no promoter hypermethylation of p21 gene was detected in all 14 cases of intestinal large B-cell lymphomas, suggesting that p21 promoter methylation is not associated with intestinal lymphomagenesis.

p27 hypermethylation was found in 9% of metastatic malignant melanoma, and p21 methylation was associated with transcriptional silencing *in situ*, suggesting that p27 methylation may be a cause of monoallelic p27 silencing in a small fraction of malignant melanomas (7). DNA methylation was correlated inversely with the expression of p27 gene products in pituitary tumor cell lines, so increased DNA methylation is an important mechanism for the silencing of p27 gene in the pituitary tumors (18). p27 hypermethylation was detected in 11% of primary central nervous system lymphomas, and 22% were negative for p27 expression (15). However, in the present study, p27 genes were unmethylated in all cases of low- and high-grade lymphomas of the stomach and intestine, suggesting that p27 promoter hypermethylation is not associated with the loss of protein expression in malignant lymphoma of the gastrointestinal tract. Because regulation of p27 can be achieved through several complementary routes including transcriptional control, altered translation (19), sequestration (20, 21), and ubiquitin-dependent degradation by proteosome (22–24), further studies are warranted to explain the molecular mechanisms for loss of p27 protein expression other than promoter hypermethylation.

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