A Cancer-prone Case with a Background of Methylation of \textit{p16} Tumor Suppressor Gene

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ABSTRACT

\textbf{Purpose and Experimental Design:} To date, the presence of \textit{p16} gene promoter methylation associated with loss of protein expression has been demonstrated frequently in digestive tract cancers. In this study, we tested for the methylation status of \textit{p16} promoter in normal tissue specimens using the methylation-specific PCR technique to examine whether \textit{p16} methylation already existed in the background of tumors.

\textbf{Results:} Aberrant promoter methylation of \textit{p16} gene was detected in 1 of 40 esophageal and 1 of 69 gastric and no colorectal epithelium specimens, and these 2 specimens were derived from the same patient. Moreover, his blood cells also exhibited the \textit{p16} methylation. These results suggested that there are cancer-prone people whose \textit{p16} gene was inactivated by methylation in normal background cells.

\textbf{MATERIALS AND METHODS}

\textbf{Sample Collection and DNA Preparation.} Forty esophageal, 69 gastric, and 99 colorectal normal epithelium specimens obtained during surgery were collected at the Nagoya University School of Medicine from each cancer patient who had been diagnosed histologically. All tissues were quickly frozen in liquid nitrogen and stored at \textasciitilde 80°C until analysis. Corresponding tumor and blood specimens were obtained from the same patients and stored at 80°C. These specimens were digested overnight by proteinase K, and DNA was prepared by extraction with phenol as described previously.

\textbf{Bisulfite Modification.} DNA from specimens was subjected to bisulfite treatment as described previously. One microgram of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp.), again treated with NaOH, precipitated with ethanol, and resuspended in water.

\textbf{MSP.} The modified DNA was used as a template for MSP. Primer sequences for amplification of \textit{p16} were described previously. The primers for the unmethylated reaction were: (a) p16UMS (sense), 5’-TTATAGGGTGGGGTGAT-TGT; and (b) p16UMAS (antisense), 5’-CAACCCCCAAAAACCA-CAACCATAA. The primers for the methylated reaction were: (a) p16MS (sense), 5’-TTATAGGGTGGGGCCGATCCG; and (b) p16MAS (antisense), 5’-GACCCCCAACCGGACCGTCAA. The PCR amplification of the modified DNA samples consisted of 1 cycle of 95°C for 5 min; 33 cycles of 95°C for 30 s, 69°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. DNA from L132 (embryonic lung cell line) and H1299 (lung cancer cell line) was amplified in parallel as positive controls.

\textbf{Conclusion:} These results suggested that the \textit{p16} gene predisposed mice to tumorigenesis, demonstrating that \textit{p16} is a tumor suppressor gene in mice. This report implied that people might be prone to generate cancers if their \textit{p16} gene has already been inactivated in normal tissues.

Received 4/8/02; revised 10/28/02; accepted 10/28/02.

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2 The abbreviations used are: MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; DNMT, DNA methyltransferase.
was used as positive controls for unmethylated and methylated alleles, respectively. Controls without DNA were performed for each set of PCR. Ten microliters of each PCR product were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Each MSP was repeated at least three times.

Bisulfite-PCR Methylation Analysis. Bisulfite-treated DNA was amplified with p16 gene-specific primers. The primers were: (a) p16F (sense), 5’-H11032-AGTTTTCGGTTGATTGGTTGG; and (b) p16R (antisense), 5’-H11032-TACAAACTTCGTCCTC-CAAAATC. The PCR amplification of the modified DNA samples consisted of 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. PCR products were digested with a restriction enzyme, Mae I, and electrophoresed on 6% acrylamide gels.

Immunohistochemistry. Immunohistochemical analysis was performed as described previously (13). The specimens were fixed with 10% formalin, embedded in paraffin, and cut into 3-μm sections in thickness, and the slides were dried at 60°C for 30 min, treated with xylene, and dehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H2O2. Microwave treatment was performed for 4 min in Antigen Retrieval Citra solution (Biogenex, San Roman, CA). After blocking with normal goat serum for 20 min, the slides were incubated with polyclonal rabbit antibody against p16 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution for 1 h at room temperature. Vectastain ABC Kit and DAB Substrate Kit (Vector, Burlingame, CA) were used to visualize the antibody binding. Only nuclear staining was regarded as positive staining. Inflammatory cells and reactive stromal cells served as positive internal controls for p16 staining.

Semiquantitative RT-PCR. First-strand cDNA was generated from total RNA. To obtain a semiquantitative result from RT-PCR, we used the minimum number of cycles required to obtain a clear signal in the linear range. The primers were: (a) p16RTS (sense), 5’-H11032-ATGGAGCCTTCGGCTGACTGG; and (b) p16RTAS (antisense), 5’-H9252-AGCACCACCAGCGTGTC. PCR amplification consisted of 30 cycles for p16 and 25 cycles for H9252-actin (95°C for 30 s, 55°C for 1 min, and 72°C for 1 min) after the initial denaturation step (95°C for 2 min). The predicted size of PCR product was 266 bp from p16. The amplification of H9252-actin cDNA demonstrated use of equal amounts of total cDNA.

RESULTS

We first examined the methylation status of p16 promoter in normal tissue specimens using the MSP technique. The aberrant promoter methylation of the p16 gene was detected in 1 of 40 (3%) esophageal, 1 of 69 (1%) gastric, and 0 colorectal epithelium specimens (Fig. 1). This result indicated that the p16 methylation of normal tissues was a rare event, as described in previous reports (14). However, we found that these two specimens were derived from the same patient, whereas other spec-
imens differed completely among each other; i.e., there were no double cancers in any patient except this one, suggesting that he may have had a predisposition to p16 methylation. His clinico-pathological data were described in Table 1.

To confirm this hypothesis, we tested for p16 promoter methylation in the corresponding tumor and blood cell DNA obtained from this patient. As expected, we also found the same methylation change in both tumor and blood cell DNA (Fig. 2). To confirm the p16 promoter methylation in these DNA samples, we performed a bisulfite-PCR methylation analysis, and all samples obtained from this patient exhibited the p16 methylation (Fig. 3). These results suggested that his p16 gene was affected by methylation to a greater or less extent.

Using immunohistochemistry, we next examined whether the methylation of the p16 gene repressed its expression in his esophageal epithelium. As shown in Fig. 4, the majority of the esophageal normal and tumor cells was negatively stained by p16 antibody in the patient with p16 methylation, whereas they were positively stained in the patient without p16 methylation. This result was in agreement with a previous report (15). We also performed a semiquantitative RT-PCR to confirm the loss of p16 gene expression in samples obtained from case E34 (Fig. 5). Taken together, we could speculate that p16 has been inactivated to a certain extent by methylation in this patient and that other additional factors may have promoted the tumor development in his esophageal and gastric tissues.

**DISCUSSION**

In a previous study, we examined the methylation status of the p16 gene in esophageal and colorectal cancer using MSP and found that 82% of esophageal and 47% of colorectal cancers exhibited p16 methylation (8, 9). This result indicated that p16 methylation may have played an important role in the tumorigenic pathway of these cancers. In this study, we tested for the methylation status of p16 in the normal tissues of these patients to examine whether p16 methylation may have already existed in the background of their tumors. Although only 1 of 207 patients with digestive tract cancers exhibited the p16 methyl-
ation, he had this aberrant methylation ubiquitously in his normal tissues, including WBCs. As for p16 methylation in normal tissues, several studies have thus far been published. Ahuja et al. (16) examined the p16 methylation in 47 samples of normal colon mucosa and observed no methylation. In fact, the p16 gene that undergoes promoter hypermethylation in cancer cells possesses unmethylated CpG islands in normal healthy cells. Thus, aberrant DNA methylation at this site can be used as a biomarker for the molecular detection of malignancy (8, 9). Therefore, it may be possible that the patient in this study at some time acquired a predisposition to p16 gene methylation and that this tended to provoke tumor formation in methylated normal tissues.

If so, how did he acquire this predisposition? One possibility was that his DNMT was abnormally activated and induced methylation in normal tissue cells ubiquitously (17). However, Eads et al. (18) examined the aberrant methylation of CpG islands in colorectal tumors and found that the frequency or extent of CpG island hypermethylation in individual tumors did not correlate with the expression of DNMT. In another study, Rhee et al. (19) described that cells lacking DNMT1 exhibited markedly decreased cellular DNMT activity but that there was only a 20% decrease in overall genomic methylation. Moreover, most of the loci that they analyzed, including the p16 gene, remained fully methylated and silenced, suggesting that CpG methylation was maintained in human cancer cells lacking DNMT1 (19). In addition, we also examined the methylation status of p14 gene in normal cells of this patient, and no DNA from his normal tissues exhibited the p14 methylation (data not shown).

Although, in this small study, we could detect only 1 patient who showed aberrant methylation in normal tissues and was prone to trigger cancers in these tissues, this is the first study describing aberrant p16 methylation in the background of cancers. Additional studies are needed using a larger population to confirm that such aberrant p16 methylation in normal tissues exists in other patients who belong to a population at high risk of developing cancers in this methylation background.

ACKNOWLEDGMENTS

We thank M. Taguchi for her technical assistance.

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