Hypermethylation of Tumor-related Genes in Genitourinary Cancer Cell Lines

Hypermethylation of CpG island is a common mechanism for the inactivation of tumor-related genes. In the present study, we analyzed 13 genitourinary cancer cell lines for aberrant DNA methylation of 5 tumor-related genes using methylation-specific polymerase chain reaction (MSP). GSTP1 was methylated in 5 (38.5%), E-cadherin in 1 (8%), VHL in 1 (8%), and MGMT and hMLH1 in none (0%). Six out of thirteen genitourinary cancer cell lines had methylation of at least one of five genes; 5 had one gene methylated, and 1 had two genes methylated. Methylation of these 5 genes was not detected in any of the bladder cancer cell lines. GSTP1 was methylated in all of the 3 prostate cancer cell lines. We conclude that aberrant hypermethylation may be an important mechanism for the inactivation of cancer-related genes in kidney and prostate cancer cell lines.

Key Words : Urogenital Neoplasms; Genitourinary cancer cell lines; HMLH1; GSTP1; E-cadherin; VHL; MGMT; Hypermethylation; Methylation-specific PCR

INTRODUCTION

Methylation of DNA is important in the genetic regulation of mammalian cells. CpG islands are GC-rich areas of the genome corresponding to the promoter regions of genes and are associated with transcriptional activity (1). The methylation status of these CpG islands has been shown to be involved with oncogene activation and tumor suppressor gene inactivation. Hypermethylation of CpG islands in the tumor suppressor gene p15INK4B, p16CDKN2, and VHL (von Hippel-Lindau) occurs frequently in various types of human malignancies (2-4).

Previous studies on the p16 gene in genitourinary cancer cell lines showed that aberrant methylation as well as genetic mutation could be an important mechanism for the inactivation of the p16 gene (5, 6). This finding raised the question whether an epigenetic component such as DNA methylation might play a role in the transcriptional silencing of other tumor-related genes in the genitourinary cancer cell lines. The question prompted us to define the role of DNA methylation in the regulation of tumor-related genes in genitourinary cancers.

A recent study on the profile of promoter hypermethylation for 12 genes (p16INK4A, p15INK4B, p14ARF, p73, APC, BRCA1, hMLH1, GSTP1, MGMT, CDMI, TIMP3, and DAPK) in 15 major tumor types revealed one or more of the genes are hypermethylated in every tumor types (7). However, the profile of promoter hypermethylation for the genes differs in each cancer type, providing a tumor type- and gene-specific profile. Transcriptional inactivation of MGMT by DNA methylation occurs in a wide spectrum of human tumors (8), whereas that of GSTP1 is characteristic of steroid-related neoplasms such as breast, liver, and prostate cancers (9, 10). Hypermethylation of the mismatch repair gene hMLH1 is restricted to the sporadic tumors with microsatellite instability (11-14). Thus, a combined methylation analysis of these three genes may contribute to develop molecular detection strategies for virtually every form of human cancers (12).

E-cadherin (E-cad) and VHL can be other candidate genes for the detection of the major human cancers. A previous study has demonstrated that the frequent loss of E-cadherin expression in human carcinomas, such as breast, prostate, and gastric cancer, results from hypermethylation of the E-cadherin promoter region (15). Mutations in the VHL tumor suppressor gene are found in 55% to 70% of clear cell renal cell carcinomas. Originally, VHL was identified in families with a VHL disease, a rare hereditary mult tumorm syndrome (16). However, recent studies defined the inactivation of VHL gene as a likely initiating event in human carcinoma (3, 17).
The identification of genes targeted by hypermethylation may provide insights into the mechanisms for the inactivation of tumor-suppressive pathways in genitourinary cancer. In addition, hypermethylated genes may serve as targets for the development of novel screening tests for cancer (18). In the present study, we examined the aberrant methylation status of five tumor-related genes in 13 genitourinary cancer cell lines by methylation-specific PCR (MSP) method.

MATERIALS AND METHODS

Cell cultures

Thirteen genitourinary cell lines were used. Their primary sites and characteristics are shown in Table 1. The cell lines T24, Caki-1, Caki-2, DU145, and LNCaP were provided by American Type Culture Collection (ATCC, Rockville, U.S.A.). The other cell lines were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). T24, Caki-1, and Caki-2 were grown in McCoy’s 5a media containing 10% heat-inactivated fetal bovine serum (FBS). Du145 was grown in MEM medium supplemented with 10% FBS, and the others were cultured in RPMI 1640 media containing 10% FBS. The cultures were kept in a 37°C, humidified chamber containing 5% CO2.

DNA isolation

Genomic DNA was obtained from these cell cultures in late log-phase growth at 75-80% confluence. Cell monolayers were washed in phosphate-buffered saline and lysed in 3 mL DNA extraction buffer (0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0). Proteinase K (20 mg/mL) was added and the samples were incubated at 50°C for 2 hr. DNA was extracted with phenol and chloroform followed by ethanol precipitation.

Bisulfite modification for MSP

DNA methylation patterns in the CpG island of the target genes were determined by chemical modification of unmethylated, but not the methylated, cytosines to uracils, and subsequent PCR using primers specific for either methylated or modified unmethylated DNA (19). Primer sequences and annealing temperatures are shown in Table 2. One microgram of DNA was denatured by NaOH and modified by sodium bisulfite.
bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), treated with NaOH again, precipitated with ethanol, and resuspended in water. Ten microliters of each PCR product was directly loaded onto 2% agarose gels, stained with ethidium bromide, and visualized by a UV transilluminator.

RESULTS

Thirteen genitourinary cancer cell lines were analyzed for the promoter hypermethylation of 5 cancer-related genes. After the bisulfite modification of DNA, MSP was employed for the detection of methylated and unmethylated DNA sequences of the target genes (20). Methylation of each CpG site of these genes was determined by the presence of unconverted cytosines. The unmethylated form of each gene was detected in each of the 13 cell lines. Significant hypermethylation of \( \text{GSTP1} \) was observed in 5 cell lines (38.5%). Hypermethylation of \( \text{E-cadherin} \) and \( \text{VHL} \) was observed in only one cell line each. No methylated templates of \( \text{MGMT} \) and \( \text{hMLH1} \) were detected by MSP in all 13 cell lines. The bands that represented the unmethylated forms, especially

Fig. 1. Methylation analyses of the promoter region CpG islands of five genes in bladder cancer cell lines by methylation-specific PCR. The cell lines are designated on the left side of each panel. The PCR products in lanes marked U indicate unmethylated genes; products in lanes marked M indicate hypermethylated genes. Lane M represents 100-bp DNA marker.

Fig. 2. Methylation analyses of the promoter region CpG islands of five genes in renal cancer cell lines by methylation-specific PCR. The cell lines are designated on the left side of each panel. The PCR products in lanes marked U indicate unmethylated genes; products in lanes marked M indicate hypermethylated genes. Lane M represents 100-bp DNA marker.

Table 3. Aberrant methylation of genitourinary cancer cell lines

| Primary sites | Cell lines | E-cad | VHL | MGMT | hMLH1 | GSTP1 |
|---------------|------------|-------|-----|------|-------|-------|
| Bladder       | T24        | U     | U   | U    | U     | U     |
|               | J82        | U     | U   | U    | U     | U     |
|               | 5637       | U     | U   | U    | U     | U     |
|               | HT-1197    | U     | U   | U    | U     | U     |
|               | HT-1376    | U     | U   | U    | U     | U     |
| Kidney        | ACHN       | U     | U   | U    | M     | U     |
|               | Caki-1     | U     | U   | U    | U     | M     |
|               | Caki-2     | U     | U   | U    | U     | M     |
|               | A-498      | M     | U   | U    | U     | M     |
|               | A-704      | U     | M   | U    | U     | U     |
| Prostate      | DU145      | U     | U   | U    | U     | M     |
|               | LNCaP      | U     | U   | U    | U     | M     |
|               | PC-3       | U     | U   | U    | U     | M     |

U, unmethylated; M, methylated
those for E-cadherin and MGMT, were faint. The detailed results of methylation for each gene in all cancer cell lines are shown in Fig. 1, 2, and Fig. 3, and are summarized in Table 2.

In the present study, 6 out of 13 genitourinary cancer cell lines showed hypermethylation of 1 to 2 genes out of 5 target genes (Table 3).

**DISCUSSION**

Methylation of the CpG islands of tumor suppressor genes leading to their transcriptional inactivation is a highly consistent feature of tumorigenesis. The studies of primary cancer cell lines indicate that methylation may constitute an alternative mechanism for silencing tumor suppressor genes.

Our data demonstrated the distribution pattern of the aberrant methylation of the tumor-related genes in genitourinary cancer cell lines. Glutathione S-transferases (GST) are a family of enzymes involved in the detoxification of xenobiotics and oxygen radicals (21, 22). Recent studies have demonstrated that the expression of the GSTP1 gene, one of the GST isoenzymes, is controlled by DNA methylation (10). In the present study, 6 out of 13 genitourinary cancer cell lines showed hypermethylation of 1 to 2 genes out of 5 target genes (Table 3). The previous studies indicate that the aberrant hypermethylation of the VHL tumor suppressor gene (17). VHL hypermethylation were identified in 13% of clear cell renal cell carcinomas (29). Another study showed hypermethylation of the VHL gene in 5 of 26 (19%) clear cell renal cell carcinomas (17). In our study, one out of 5 renal cancer cell line (1/5, 20%) showed hypermethylation of the VHL gene.

Expression of the homotypic cell-to-cell adhesion molecule, E-cadherin, suppresses tumor cell invasion and metastasis in experimental tumor models. It has been demonstrated that transcriptional inactivation of E-cadherin expression occurs frequently during tumor progression, and that E-cadherin expression in human cancer cells is regulated by CpG methylation around the promoter region. A previous study showed that the loss of E-cadherin function contributes to progression of solid tumors such as breast (30) and gastric cancers (31). In the present study, only one renal cancer cell line showed hypermethylation of this gene.

The DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) removes alkyl adducts from a methyl group to an active cytosine in its own sequence through a reaction that inactivates the MGMT molecule itself for each lesion repaired. The loss of expression of MGMT is rarely due to genetic mutation, but due to methylation of discrete regions of the CpG island of the gene has been associated with the silencing of the gene in primary human neoplasia (8) including brain, head and neck, gliomas, lung and colorectal carcinomas. The tumors with silenced MGMT by aberrant methylation include those with a frequent rate of K-ras mutation, such as colon, lung, and head and neck carcinomas (32). This suggests that one potential consequence of the loss of MGMT expression may be an increased susceptibility to K-ras mutation. Bladder carcinomas are known to be associated with a carcinogen exposure. However, in the present study, aberrant hypermethylation of the MGMT promoter region was not detected in any of the genitourinary cancer cell lines.

The previous studies indicate that the aberrant hypermethylation of the hMLH1 promoter and the consequent transcriptional silencing is a common event in the formation of sporadic microsatellite unstable colon cancer (33). Others have demonstrated a strong correlation between the presence of hMLH1 hypermethylation and MSI+ tumors in colorectal (12, 34), endometrial (13, 35), and gastric tumors (36), and an absence of hMLH1 methylation in other tumor types (13). The accurate proportion of hypermethylation of hMLH1 in genitourinary cancer has not been determined. In the present study, none of the 15 genitourinary cancer cell lines showed hypermethylation of hMLH1 promoter region.

Southern hybridization approaches reveal overall methylation status of CpG islands, but can only provide information about those CpG sites within the sequences recognized by methylation-sensitive restriction enzymes (37). A more
sensitive method of methylation-sensitive restriction enzyme digestion followed by PCR is prone to false-positive results, since any uncleaved DNA will be amplified by PCR to yield a positive result for methylation. The chemical modification of cytosine to uracil by bisulfite treatment and direct sequencing is not only technically difficult but also labor-intensive and less sensitive than Southern analysis. Recently described bisulfite-based PCR method called MSP is an excellent alternative, but it is usually a qualitative, rather than quantitative method. Several investigators have developed genome-scanning techniques sensitive to DNA methylation to gain appreciation of the genome-wide changes occurring within various cancers (38). These quantitative MSP can be applied to elucidate diverse biological processes involving DNA methylation and therefore is believed to provide more accurate information about the effect of aberrant methylation on carcinogenesis.

From the results of the present study, we conclude that aberrant hypermethylation may be a common mechanism to inactivate cancer-related genes in kidney and prostate cancer cell lines. The exact nature of the methylation defect in cancer cells should be defined by further studies employing quantitative MSP.

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