J Korean Med Sci 2001; 16: 756-61
ISSN 1011-8934

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*

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Received : 28 June 2001
Accepted : 27 August 2001

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*This research was supported by Korea Science and Engineering foundation (KOSEF) through the Biomolecular Engineering Center at Kyungpook National University.

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 $p15^{INK4B}$, *p16/CDKN2*, 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 ($p16^{INK4A}$, $p15^{INK4B}$, $p14^{ARF}$, *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 develope 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 multitumor 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℃, humidified chamber containing 5% CO_2.

Table 1. Primary sites and histopathology of the cell lines

Cell line	Primary site	Histopathology
T24	Bladder	Transitional cell carcinoma
J82	〃	Transitional cell carcinoma
5637	〃	Carcinoma
HT-1197	〃	Carcinoma
HT-1376	〃	Carcinoma
ACHN	Kidney	Renal cell adenocarcinoma
Caki-1	〃	Clear cell carcinoma: metastasis to skin
Caki-2	〃	Clear cell carcinoma
A-498	〃	Carcinoma
A-704	〃	Adenocarcinoma
DU145	Prostate	Brain metastasis
LNCaP	〃	Lymph node metastasis
PC-3	〃	Bone metastasis

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℃ 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

Table 2. PCR primers used for methylation-specific PCR

Primer set	M/U	S/AS	Sequence (5′→3′)	Size (bp)	Annealing temp. (℃)
E-cad	M	S	TTAGGTTAGAGGGTTATCGCGT	115	57
		AS	TAACTAAAAATTCACCTACCGAC		
	U	S	TAATTTTAGGTTAGAGGGTTATTGT	97	53
		AS	CACAACCAATCAACAACACA		
VHL	M	S	TGGAGGATTTTTTTGCGTACGC	158	60
		AS	GAACCGAACGCCGCGAA		
	U	S	GTTGGAGGATTTTTTTGTGTATGT	165	60
		AS	CCCAAACCAAACACCACAAA		
MGMT	M	S	TTTCGACGTTCGTAGGTTTTCGC	81	59
		AS	GCACTCTTCCGAAAACGAAACG		
	U	S	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93	59
		AS	AACTCCACACTCTTCCAAAAACAAAACA		
hMLH1	M	S	ACGTAGACGTTTTATTAGGGTCGC	112	60
		AS	CCTCATCGTAACTACCCGCG		
	U	S	TTTTGATGTAGATGTTTTATTAGGGTTGT	124	60
		AS	ACCACCTCATCATAACTACCCACA		
GSTP1	M	S	TTCGGGGTGTAGCGGTCGTC	91	55
		AS	GCCCCAATACTAAATCACGAC		
	U	S	GATGTTTGGGGTGTAGTGGTTGTT	97	55
		AS	CCACCCCAATACTAAATCACAACA		

M, methylated; U, unmethylated; S, sense strand; AS, antisense strand

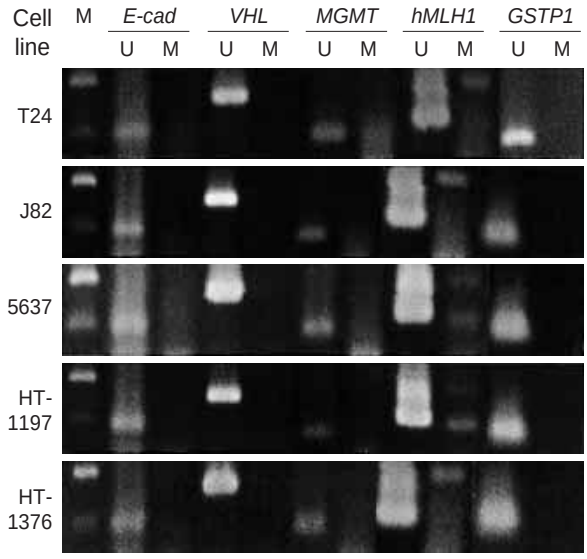

Fig. 1. Methylation analyses of the promoter region CpG islands of five genes in bladder cancer cell lines by methylation-specific PCR. The cell lines are designated on the left side of each panel. The PCR products in lanes marked U indicate unmethylated genes; products in lanes marked M indicate hypermethylated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

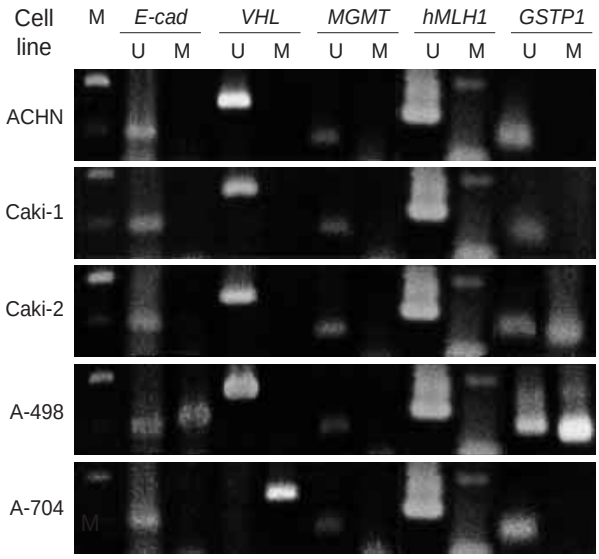

Fig. 2. Methylation analyses of the promoter region CpG islands of five genes in renal cancer cell lines by methylation-specific PCR. The cell lines are designated on the left side of each panel. The PCR products in lanes marked U indicate unmethylated genes; products in lanes marked M indicate hypermethylated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

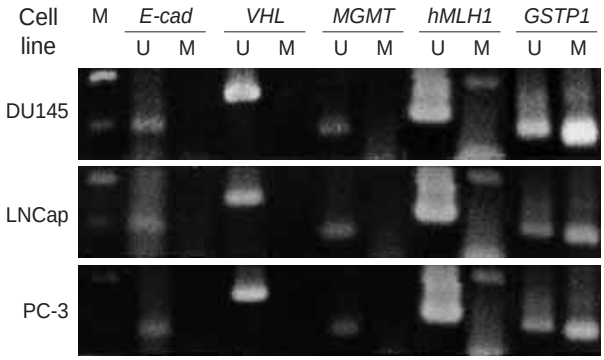

Fig. 3. Methylation analyses of the promoter region CpG islands of five genes in prostate cancer cell lines by methylation-specific PCR. The cell lines are designated on the left side of each panel. The PCR products in lanes marked U indicate unmethylated genes; products in lanes marked M indicate hypermethylated 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	U
	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

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 *GSTP1* was observed in 5 cell lines (38.5%). Hypermethylation of *E-cadherin* and *VHL* was observed in only one cell line each. No methylated templates of *MGMT* and *hMLH1* were detected by MSP in all 13 cell lines. The bands that represented the unmethylated forms, especially

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). Hypermethylation of the *GSTP1* promoter region was found in 75% of mostly localized tumors (23). Quite unexpectedly, the promoter region of the *GSTP1* gene, which is unmethylated in normal prostate and benign hyperplasia, was found to be hypermethylated in carcinomas (24, 25). This finding is intriguing because *GSTP1* is not considered a tumor suppressor gene. Whereas the other members of the family become downregulated during tumorigenesis (26, 27), GSTP1 is the most ubiquitously expressed isoenzyme and even overexpressed in some other cancers. The recently developed technology of gene expression arrays has been used to study the gene expression profile of solid tumors. It appears that the gene expression profile, which includes the type of genes expressed and the level of expression, is altered in neoplasms. Of the genes that yielded significant signals, *GSTM1* showed a greater reduction in mRNA level in prostate cancer specimens than in normal prostate specimens (28). This result suggests that GST-based metabolism is potentially important in cancers, and deserves further investigation. In our study, 3 out of 5 kidney cancer cell lines and all of the 3 prostate cancer cell lines showed *GSTP1* promoter hypermethylation, suggesting hypermethylation of *GSTP1* is an important factor for kidney and prostate tumorigenesis. Hypermethylation of the *GSTP1* gene in primary renal cancer tissue has not been reported previously, and further studies with primary renal cancer samples are needed.

Gnarra et al. studied whether the changes of methylation status in the CpG island of the *VHL* gene accounted for the loss of expression of the gene (17). They observed a renal cell culture line treated with 5-aza-2′-deoxycytidine resulted in a re-expression of the VHL gene (17). To elucidate the role of somatic alterations for the etiology and prognosis of renal cancers, 227 sporadic renal epithelial tumors were analyzed for mutations and hypermethylations in the *VHL* tumor suppressor gene (3). *VHL* hypermethylations were identified in 13% of clear cell renal cell carcinomas (29). Another study showed hypermethylation of the 5′ region of this 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 O^6-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 13 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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