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Hypermethylation of the Putative Tumor-suppressor Genes DCC, p51/63 and O^6-methylguanine-DNA Methyltransferase (MGMT) and Loss of Their Expressions in Cell Lines of Hematological Malignancies

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Abstract

Alterations and defective expression of three putative tumor-suppressor genes, the deleted in colorectal cancer (DCC), p51, and O^6-methylguanine-DNA methyltransferase (MGMT), have been demonstrated in many cancers. However, it is not known whether the defective expression of each of these genes is independent or whether it reflects a specific methylation abnormality. Here, we investigated the expression of the DCC, p51 and MGMT genes and the methylation status of the 5' flanking CpG region in 17 cell lines established from hematological malignancies. The reverse transcriptase-polymerase chain reaction method showed DCC expression to be absent in 13 of the 17 cell lines and showed expression of both p51 and MGMT to be absent in 5 of the 17 cell lines. The methylation patterns were analyzed with methylation-specific polymerase chain reaction (MSP) of the 5' flanking region of the DCC and p51 genes and the promoter region of the MGMT gene. Although unmethylated patterns of the CpG region in the DCC, p51, and MGMT genes were observed in all 11 normal controls, abnormal methylation patterns of these genes were found even in many cell lines expressing these genes. A hypermethylation pattern was detected for the CpG region of MGMT and p51 in cells that did not express these genes. In contrast, a hypermethylation pattern was not always detected for the CpG region of DCC in cells with reduced DCC expression. The results of this study indicate that in many hematological cell lines, the DCC, p51, and MGMT genes have been abnormally methylated in the CpG region. Hypermethylation of these three genes may be independent events in each cell line.
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Key words: deleted in colorectal cancer, p51/p63, O^6-methylguanine-DNA methyltransferase, methylation, loss of expression

Introduction

The development of human cancers, including hematological malignancies, is associated with an accumulation of genetic alterations of oncogenes and tumor suppressor genes. Several tumor-suppressor genes or candidates, including p53[1–3], p73[4], $p16^{ink4,5}$, $p15^{ink4b,6}$, and FHIT[7], have been reported to be mutated or deleted in hematological malignancies. The expressions of these genes have also been reported to be inactivated. The mechanism of $p16^{ink4}$

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and $p15^{ink4b}$ inactivation is reported to be aberrant methylation of the CpG island promoters[8], which has been shown in several by human cancers. Hypermethylation is thought to be the major mechanism of inactivation of these genes[9].

Loss of heterozygosity on chromosome 18q21 is frequently found in many cancers[10–12], and the deleted in colorectal cancer (DCC) gene has been postulated to be the involved gene. However, few reports have focused on DCC gene mutations[13], probably because of the gene's length and complexity[14]. The p51 gene, a member of the p53 gene family, was recently discovered. Like p53, p51 activates the transcription of $p21^{Walf/Cipl}$ and BAX, and induces apoptosis[15–18]. Thus, p51 is thought to be a tumor-suppressor gene. Only a few reports have focused on p51 gene mutations[19]. The expression of O^6-methylguanine-DNA methyltransferase (MGMT) is decreased in some cancers[20]. Methylation of the MGMT promoter in *de novo* cancers is associated with loss of MGMT expression[21–23]. The relationship between the expression and methylation status of the DCC and p51 genes remains unclear, as is whether the defective expressions of these genes are independent events.

Here, we investigated the methylation status of the CpG region and the expression of the DCC, p51 and MGMT genes in various cell lines established from hematological malignancies. We also analyzed the relationship between defective gene expression and the defective methylation status of these three genes.

Materials and Methods

Cell Lines

Seventeen cell lines established from various hematological malignancies, consisting of 5 chronic myelogenous leukemia (CML) cell lines (K562, SAS 413, KT-1, NCML, KU812), 6 acute myelogenous leukemia (AML) cell lines (YSK21[24], OE, HL60, HEL, OE-R, JIII), 3 acute lymphocytic leukemia (ALL) cell lines (M13, U937, OM9;22) and 3 malignant lymphoma cell lines (TK[25], Raji, KML-1[26]), were used. OE and OE-R were cell lines newly established from AML (M0) patients in our laboratory. OE and OER cells were established from bone marrow obtained at diagnosis and relapse. Phenotypically, OE cells were positive for only CD33 and negative for CD2, 3, 4, 7, 8, 13, 34, 117 and HLA-DR. The OER cells were positive for CD33 and 117 and negative for CD2, 3, 4, 7, 8, 13, 34, and HLA-DR. Both cells had complex karyotipic abnormalities. Chromosome 18 was normal in both cells. Molecular genetic analysis revealed that both cells showed a p53 mutation at codon 168 (CAC→CGC). No mutation of the N-ras gene was detected. As normal controls, bone marrow mononuclear cells (BM-MNCs) from 11 healthy volunteers were used.

Gene-expression Analysis with the Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

Gene expressions of MGMT, p51 and DCC were analyzed with RT-PCR. Total RNA was extracted from the cell lines and normal BM-MNCs with an RNAzol Extraction Kit (Biotex Laboratories, Houston, TX, USA) according to the manufacturer's inmstructions. Using 1 μg of total cellular RNA to generate cDNA with random 9mer primers and RT (Takara Shuzo, Kyoto, Jpn), the cDNA was amplified by PCR with primers. The sequences of the PCR primers used were as follows. MGMT: 5'-GCCGGCTCTTCACCATCCCG-3' (upper primer) and 5'-GCTGCAGACCACTCTGTGGCACG-3' (lower primer)[6]; p51; 5'-AAAGAAAGTTATTACCGAT-3' (upper primer) and 5'-CGCGTGGTCTGTGTTATAGG-3' (lower primer)[19]; and DCC: 5'-TTCCGCCATGGTTTTTAATCA-3' (upper primer) and 5'-AGCCTCATTTTCAGCCACACA-3' (lower primer)[27]. After PCR, 10 μ*l* of each sample was electrophoresed in 2.0% agarose gels and visualized by ethidium bromide staining. The RT-PCR of β-actin served as an internal control.

Gene-methylation Analysis with Methylation-specific PCR (MSP)

Total cellular DNA was extracted with protease K digestion, phenol-chloroform extraction and ethanol precipitation[28]. DNA was also extracted from BM-MNCs as a normal control. The DNA methylation patterns were analyzed with MSP, as described

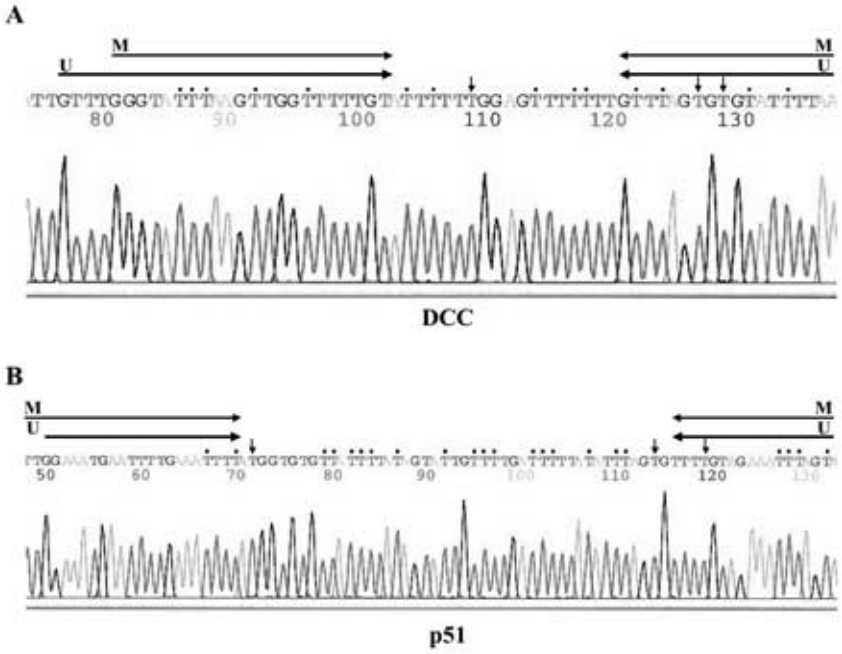

Fig. 1 Sequence analysis of the cloned MSP product of the unmethylated MSP product of the DCC gene (A) and the unmethylated MSP product of the p51 gene (B) from BM-MNCs DNA. Ts with a dot indicate thymine conversion from cytosine by bisulphite reaction. Ts with an arrow indicate unmethylated cytosine residues of the CpG region.

previously[24]. The DNA methylation patterns in the CpG island of the MGMT, p51, and DCC genes were determined by chemical modification of unmethylated, but not methylated, cytosines to uracil and subsequent PCR using primers specific for either methylated or modified unmethylated DNA[29]. The primer sequences of MGMT, p51 and DCC for the unmethylated reaction were 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (upper primer) and 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (lower primer), 5'-GGAAATGAATTTTGAAATTTT-3' (upper primer) and 5'-ACTAAATTTCTACAAAA-3' (lower primer), and 5'-GTTTGGGTATTTAAGTTGGTTTTTGTA-3' (upper primer) and 5'-AAAATACACACTAAAC-3' (lower primer), respectively. For the methylated reaction, the sequences were 5'-TTTCGACGTTCGTAGGTTTTCGC-3' (upper primer) and 5'-GCACTCTTCCGAAAACGAAACG-3' (lower primer), 5'-TTGAAGGAAATGAATTTTGAAATTTT-3' (upper primer) and 5'-ACTAAATTTCTACGAAA-3' (lower primer), and 5'-GGGTATTTAAGTTGGTTTTTGTA-3' (upper primer) and 5'-AAAATACGCGCTAAAC-3' (lower primer), respectively. Sss-I methylase-treated DNA and normal BM-MNC DNA served as methylation-positive and -negative controls after bisulfite modification.

Briefly, 1 μg of DNA was denatured with NaOH and modified with sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin, treated again with NaOH, precipitated with ethanol, and resuspended in water. Ten-microliter samples of each PCR reaction product were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. To determine DNA sequences of the products of methylated and unmethylated MSP, they were subcloned into the EcoRV site of the pGEM-5Zf (+/−) cloning vector, and amplified with PCR using a T7 or SP6 primer. The MSP products of five clones were sequenced using an ABI sequencer with dye terminators (Perkin-Elmer, Warrington, U.K.). **Fig. 1** shows the unmethylated MSP products of the DCC and p51 genes.

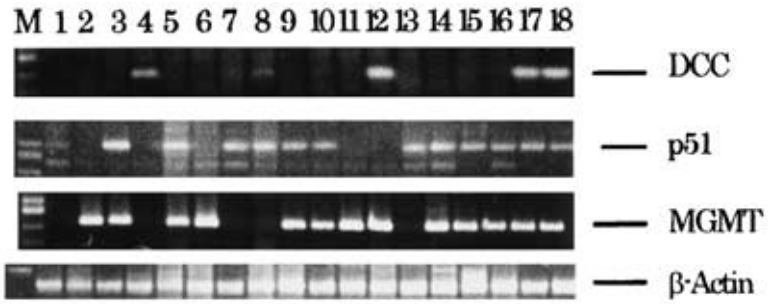

Fig. 2 Expression analysis of DCC, p51 and MGMT by RT-PCR
1: K562, 2: KML-1, 3: YSK22, 4: SAS413, 5: OE, 6: M13, 7: HL60, 8: U937, 9: HEL, 10: KT-1, 11: OM9;22, 12: OER, 13: Raji, 14: J-III, 15: TK, 16: NCML, 17: KU812, and 18: normal BM-MNCs. β-actin was used as an internal control. M shows a DNA fragment molecular marker of HaeIII-digested ϕx DNA.

Results

Gene Expression Analysis

The MGMT, p51 and DCC genes were expressed in all 11 normal controls (data not shown). In contrast, defective MGMT gene expression was observed in 5 cell lines (K562, HL60, U937, Raji, and SAS413), while defective p51 expression was observed in five cell lines (KML-1, SAS413, M13, OM

Table 1 Summary of DCC, MGMT, and p51 genes expression and methylation

	Leukemia type	DCC expression	DCC methylation		MGMT expression	MGMT methylation		P51 expression	P51 methylation	
			M[a]	U[b]		M	U		M	U
K562	CML	N[c]	+[d]	+	N	+	−[c]	P	+	+
TK	ML	N	+	±[d]	P[e]	+	+	P	+	+
YSK21	AML	N	+	+	P	−	+	P	±	±
SAS413	CML	P	−	+	N	+	−	N	+	+
OE	AML	N	+	±	P	+	+	P	+	+
M13	ALL	N	+	−	P	+	+	N	+	±
HL60	AML	N	+	±	N	+	−	P	±	±
U937	ALL	±	+	+	N	+	−	P	+	+
HEL	AML	N	+	±	P	−	+	P	−	+
KT-1	CML	N	+	+	P	+	+	P	+	+
OM9;22	ALL	N	+	±	P	ND[f]	ND	N	+	+
OE-R	AML	P	+	+	P	+	+	N	+	±
Raji	ML	N	+	±	N	+	−	P	+	±
JⅢ	AML	N	+	+	P	+	+	P	ND	ND
KML-1	ML	N	+	+	P	−	+	N	+	±
NCML	CML	N	+	+	P	+	+	P	+	+
KU812	CML	P	−	+	P	+	±	P	+	+
Controls[g]		P	−	+	P	−	+	P	−	+

[a] methylation-specific PCR
[b] unmethylation-specific PCR
[c] negative
[d] weak positive
[e] positive
[f] not done
[g] 11 normal BM-MNCs

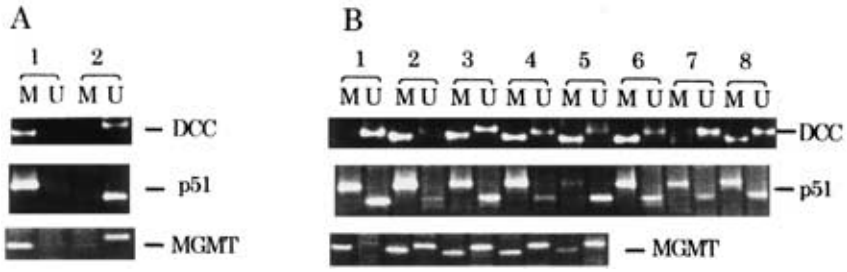

Fig. 3 Methylation analysis of DCC, p51, and MGMT with MSP
(A) Lane 1: DNA from normal BM-NMCs methylated with SssI. Lane 2: DNA from normal BM-MNCs before methylation with SssI. M: MSP with methylation-specific PCR primer, U: MSP with unmethylation-specific PCR primer. (B) Lane 1: KU812, Lane 2: M13, Lane 3: KT-1, Lane 4: OER, Lane 5: HEL, Lane 6: TK, Lane 7: SAS413, Lane 8: NCML, M: MSP with methylation-specific PCR primer, and U: MSP with unmethylated-specific PCR primer.

9;22 and OE-R) (**Fig. 2**). DCC expression was detected in OE-R, SAS413, and KU812, decreased in U937, and was absent in the other cell lines (**Fig. 2**).

Methylation Analysis

Table 1 summarizes the gene expression and methylation results. Methylation patterns of the 5' flanking region of the DCC and p51 genes and the promoter region of the MGMT gene were analyzed with MSP (**Fig. 3**). Unmethylated patterns of the CpG region in the DCC, p51 and MGMT genes were observed in all 11 normal BM-MNCs (**Table 1**). In contrast, the same degree of methylation patterns of these genes was found in all of the cell lines expressing these genes (**Fig. 3**). In MGMT, hypermethylation of the promoter region was observed in 5 cell lines without gene expression, but in the other cell lines with MGMT expression the promoter region was unmethylated or predominantly unmethylated (**Fig. 3**). In p51, unmethylation of the 5' flanking region was also observed in all normal BM-MNCs, whereas in the cell lines without p51 expression, the 5'-flanking region was methylated or predominantly methylated. In DCC, the hypermethylation pattern was detected in the CpG region of DCC in many cells without DCC expression (**Table 1**). In OE-R cells, both status of bands were co-existent. In KU 812, the 5'-flanking CpG region of DCC was unmethylated.

Discussion

It has been demonstrated that inactivation of putative tumor-suppressor genes, such as p16, p15, p73, and hMLH1[30] is associated with hypermethylation in the CpG island of the promoter regions. The DCC gene is known as a tumor-suppressor gene, and it has been reported that expression of this gene is decreased in 10% to 30% of *de novo* hematological malignancies[27, 31]. Recently, the methylation status of the DCC gene was reported to be significantly correlated with loss of DCC expression in gastric cancer[32]. Thus, in the present study, we analyzed the correlation between loss of DCC expression and hypermethylation of the 5'-flanking region near promoter regions. Because the promoter sequence of DCC has not yet been identified, we designed primers flanking the start codon for MSP. Methylation originates in either flanking region and spreads to include the CpG island near the transcriptional start site, exon 1[33]. Our results showed that although expression levels, as shown with RT-PCR, are related to methylation patterns, a hypermethylation pattern was not always detected in cells without DCC expression, and the methylation pattern did not seem to be associated with the amount of mRNA. This discrepancy between the methylation status and DCC expression might have resulted from differences in the methylation status between the promoter CpG islands and the 5' flanking regions we studied. Also,

other mechanisms that interfere with DCC expression might be involved in hematologically malignant cells. In KML-1 cells, in which loss of DCC expression was detected, an unmethylated pattern was detected in the CpG island. A possible explanation for this discrepancy is that a mutation may occur in the promoter region of the DCC gene.

p53 is an essential tumor-suppressor gene which monitors the cell cycle at the G1 check point and induces apoptosis under conditions of DNA damage and stress[34, 35]. p53 is also the gene most frequently mutated in several types of human tumors[36]. The novel p51 gene, which possesses significant amino-acid sequence similarities with p53, was discovered recently. p53 was identified on 3q28 and reportedly cooperates with p53 in tumor suppression[15]. Because the promoter sequence of p51 has not yet been identified, we designed primers flanking the start codon for MSP. Methylation originates in either flanking region and spreads to the CpG island near the transcriptional start site, exon 1[33]. In our present study, 4 cell lines revealed a relationship between the lack of expression of p51 and a hypermethylation pattern in the 5'-flanking region (**Table 1**). In contrast, in the other cell lines showing p51 expression, the 5'-flanking regions were unmethylated or predominantly unmethylated. These results suggest that hypermethylation might be the predominant mechanism causing inactivation of the p51 gene in hematological tumorigenesis. After the sequence of the promoter region of the DCC gene or the p51 gene will be clear, methylation analysis of the promoter region would be needed.

MGMT is a repair protein that specifically removes promutagenic alkyl groups from the O^6 position of guanine in DNA[37]. Repair of O^6-alkylguanine adducts by tumor cells has been implicated in drug resistance because it reduces the cytotoxicity of alkylating chemotherapeutic agents[38]. Esteller *et al*. have also reported that epigenetic silencing of MGMT by promoter hypermethylation may lead to a particular genetic change in human cancer, specifically G-to-A transitions in the K-ras oncogene[39]. Thus, MGMT would act as a tumor-suppressor gene. We found loss of expression of MGMT in 5 of the 17 tested cell lines and a hypermethylation pattern in 15 cell lines. These results suggest that inactivation of MGMT might also play a definite role in carcinogenesis.

We analyzed the association between the methylation status of the CpG region of 3 putative tumor-suppressor genes and the expression of these genes in 17 cell lines established from hematological malignancies. In the present leukemic cell lines, the methylation status of these genes correlated significantly with loss of their expression, but hypermethylation of these genes seemed to occur independently. From the viewpoint of carcinogenesis, aberrant DNA methylation of these putative genes serves as an alternative mechanism for the proliferation of tumor cells. In recent years, it has become clear that alterations of the methylation pattern of DNA are common in cancer cells and are capable of directly modifying carcinogenesis. Although, methylation may be a major mechanism in multistep carcinogenesis, further investigation of DNA methylation will be needed to clarify its role in carcinogenesis.

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