Variable Expression and Hypermethylation of p16 Gene in Patients with T-ALL and Cell Lines

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ABSTRACT

The multi tumor suppressor genes MTS1 (CDKN2, p16INK4A) and MTS2 (CDKN1, p15INK4B) located at 9p21-22 are inactivated in some human cancers via several mechanisms including deletion and hypermethylation. In hematological malignancies, deletion of p16/p15 locus has been shown to be highly specific to lymphoid malignancies, and more particularly to T-cell acute lymphoblastic leukemia (T-ALL). We have investigated the deletion, methylation and p16 protein expression status of MTS1 in T-cell childhood acute lymphoblastic leukemia (19 cases) and cell lines[11]. On Southern blot homozygous deletions or hemizygous deletion with rearangement were detected in 4/19 T-ALL. The expression of p16 protein was not observed on Western blot in 4/15 T-ALL with intact p16 gene. The p16 gene was methylated 3/15 in T-ALL. Only one of three expressed p16 protein. The other 11/15 T-ALL had p16 protein expression but different level. Loss of MTS1 was observed in 3/11 cell lines. Cell line with MTS1 gene had p16 protein expression in 6/8. After treatment with the demethylating agent (5-AzoCyt) RD cell line showed p16 expression. This has not been observed with the other cell lines. Thus hypermethylation of MTS1 is rare in childhood T-ALL. Although inactivation of MTS1 by deletion is common in T-ALL and cell lines. Furthermore our data show that the p16 gene inactivation by hypermethylation and deletion may play a role in the leukemogenesis.

Key Words: Tumor suppressor gene, p16, Acute lymphoblastic leukemia (ALL), Methylation, Expression.

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INTRODUCTION

Development of cancer is generally regarded as a process in which cumulative mutations of oncogenes and tumor suppressor genes lead to unrestrained neoplastic growth. The past several years, cyclin dependent kinases (CDKs) which are a new class of small proteins, possibly involved in the negative regulation of the cell cycle, were isolated and extensively characterized[1,2]. Recently, proteins of a new function class have been identi-

fied which inhibit the CDK activity but which are distinct from the kinases that phosphorylate and inactivate CDKs. Such proteins are called CDIs. These proteins are of interest because of the negative regulation of the CDK activities at appropriate time points in the cell cycle. These genes were shown to represent families of proteins. The mammalian CDIs up to date include p21, p27, p28, p20, p16, p15 and p18[3,4].

A high frequency of loss of heterozygosity on chromosome 9p21 has suggested the presence of one or more tumor suppressor genes in this region. Datas to date suggest that the gene involved in this region is p16. Homozygous deletion is a frequent mechanism for inactivation of this putative tumor suppressor gene in cell line and ALL patients[5]. The p16 gene is comprised of three coding exons consisting of 126, 307, and 11 base pairs, respectively. Mutations are most common in exon 2. Mutated p16 cannot inhibit CDK4 kinase activity and cells in which p16 was mutated or deleted would be expected to have a growth advantage[6-8]. The p16 protein inhibits CDK4 and CDK6, which are key regulatory factors for the progression of eukaryotic cells through the G1 phase of the cell cycle[9].

The p15/MTS2/INK4B and the p16/MTS1/INK4A genes have been reported to be altered in many types of cancers such as 40-50% homozygous deletion or point mutation were observed in primary breast carcinomas and 19% of bladder tumors and 37% of panctreatic carcinomas[10-12]. In hematological malignancies, the higher frequency of p15 del and p16 del were seen in acute lymphoblastic leukmia (> 30%) with striking rates in T-ALL (> 50%), but also low rates in B-cell precursor (BCP)-All (20%)[2,13,14].

Transcription repression by DNA-methylation of promoter and 5' regulatory sequences may be a pathway to inactivate the p16 CDKN2 and p15INK4b genes. Global changes in DNA methylation patterns are known to occur during tumorgenesis, and gene silencing has been associated with methytation of CpG islands located in or nearby promoters and 5' regulatory regions[15,16]. CpG islands are G + C rich regions that show a higher frequency of CpG dinucleotides that is normally seen in the vertebrate genome and that are not methylated in the germline. Widespread methylation of CpG island occurs on autosomal genes during oncogenic transformation[15,17]. Promoter silenced by methylation can be reactivated in many cases by treatment with the drug 5-azo2'-deoxycytidine (5-AzoCyt), which is a well known inhibitor of DNA methylation[18,19]. Methylation of p16 were found 50% of ALL cell lines, 50% of AML cell lines and 38% AML patients[20,21].

We hypothesized that abnormal DNA methylation might be an alternative mechanism for inactivation of the p16 gene in T-ALL and cell lines.

MATERIALS and METHODS

Patients, Cell Lines and Normal Controls

We previously analyzed 27 cell lines including two AML cell lines for homozygous deletions of exons CDKN2 by Southern blot analyzing[22]. 93-27, U937, RD, 93-10, EI, AE, 697, HL-60 cell lines which have p16 gene and p16 mRNA, have been selected for this research and 91-06, REH and 855 cell lines which have p16 deletion also chosen as a negative control in this research. The phenotypes of most of these cell line have been previously described, most have B-ALL phenotype. 93 10 (EU-9) has T-ALL phenotype. One myeloid cell line HL-60 and one non-Hodgkin's lymphomas cell line, U937, was also examined in this study. Bone marrow or peripheral blood samples were collected from 19 patients with T-ALL. In all examined samples, the proportion of leukemic cell exceded 80% mononuclear cells were separated from the samples on Ficoll-Hypaque density gradient, suspended and kept at -80°C. Also 6 hematologically normal bone marrows were analyzed as positive control.

Southern Blotting

Genomic DNA was extracted from cell lines, patients and hematologically normal cases by proteinase K/detergent digestion, phenol-chloroform extraction and ethanol precipitation. Seven micrograms of genomic DNA was digested with HIND III and SMA I for overnight at 37°C, then se-

parated by a 0.8% agarose gel electrophoresis; denatured; transferred to Nybond-membrane (Amersham, Buckinghamshine, UK) and fixed by baking in a vacuum oven at 80°C for 2 h. These DNA's were prehybridized for 1 h at 65°C in a solution containing 6 x SSC, 10 x Denhardt's, 1% SDS and 100 µg/mL denatured ssDNA and then hybridized overnight at 65°C in the same solution and previously labeled with p32 by the random priming technique probe which derived from either p16 exon one and exon two[22].

Analysis of Methylation of the 5'CpG Island of p16 Gene in Cell Lines

The cell lines were maintained in RPMI-1640 with 10% fetal calf serum for the study of demethylation and they were treated with 1.0 µm 5-azo-2-deoxycytidine. The drug was dissolved in cold DMEM immediately before use. The cells were incubated for 3 days with the medium and drug being replaced every 24 h. DNA and protein were harvested immediately following drug expression. DNA was extracted and Southern blotting performed according to the method described above.

Western Blot Analysis

Protein samples, equivalent to 10^6 cells were isolated using lysine buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) nonidet p-40; 1 mM phenylmethylsulfonyl fluoride, 20 µg/mL aprotinin, and 25 µg/mL leupeptin for 1 h at 4°C. The cell debris were removed by centrifugation at 14.000 rpm at 4°C for 1 h. Supernatants were collected and the protein content of the lysate was determined by the Brodford protein assay (BIO-RAD) according to the manufacturer instructions. Ten micrograms of total cellular protein was run per lane on a 12.5% SDS-polyacrylamide gel and transferred to Nitrocellulose (BIO-RAD). Membrane was blocked 30 min in 5.0% dry milk, 10 mM Tris-HCI pH 8.0, 150 mM NaCl, 2.0% BSA, 0.02% NaV3. The blocked membrane was then incubated with the monoclonal antibody anti p16, clone G175.405 (Pharmingen San Giego, CA) used 0.2 µg/mL overnight at 4°C. followed by three washes in 10 mM Tris-HCL, pH 8.0, 150 mM NaCl and 0.05% Tween 20 for 15 min each. The secondary antibodies was a 1:2500 dilution of peroxidase-conjugated anti-mouse (Dako Ltd, High Wycombe, UK) as appropriate. Detection was enhanced chemiluminescence (ECL: Amersham, UK) according to the manufacturer's recommendations. For repeated analysis of protein filters, previously bound antibody complexes were stripped from membranes by incubating then for 30 min in 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris HCL (pH 6.7) at 50°C. The blots were reprobed for actin as a control for protein loading and integrity.

RESULTS

Analysis of Methylation in T-ALL Patients

A total of 19 T-ALL patients were included in p16 gene methylation analysis. The restriction map of p16 gene was given below (Figure 1). DNA from all patients were digested with HIND III and SMA I and then hybridized with a 5' p16/CDKN2 probe. Digestion with HIND III and SMA I resulted in 6 kb band on Southern blot (Figure 2). The results indicate that 5CpG island of the p16 gene was methyleted and resulted 1 kb, 0.5 kb and 0.3 kb indicate unmethylation of p16 gene. Homozygous deletion of p16 gene was found in four cases, all from T-ALL samples (4/19;21%) (Table1). The majority of p16/CDK2 CpG islands in 12/15 patients samples were unmethyleted (80%) that have wild p16 gene. Only 3/15 patients were methylated (Table 1, Figure 1).

Analysis of p16 Methylation and Expression in Cell Lines Treated With 5-aza-2-deoxcycytidine

No significant change in the level of p16 was observed in 7 cell lines expressing p16 protein, before and after 5-azoCyt treatment. In 93-27 and EI cell lines have high, 93-10, 697, U937 and AR cell lines have love level p16 protein expression were observed according to the normal control. RD cell line did not show p16 protein expression before 5azoCyt but it did express after 5azoCyt treatment. However HL-60 cell lines has no detectable p16 protein (Table 2).

p16 Protein in ALL Patients

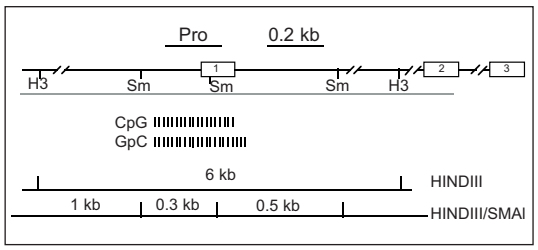

Figure 1. The restriction map and representation of the p16 gene. Pro is the probe used for Southern analysis. Boxes in the line are exons 1-3. A restriction map including the flanking enzymes HIND III (H3) and the position of the methylation-sensitive restricyion enzyme site used to determine the methylation status of this CpG island, including SMA I (sm) is also shown. The density of CpG and CpG dinucleotides are shown below the gene, and predicted restriction frangments for enzymes in the study are depicted at bottom.

Protein samples of 15 T-ALL patients which have p16 gene and six control were analyzed by Western blotting using monoclonal anti p16 antibody. 11/15 (74%) patients express p16 protein in detectable level. The three samples in which p16 was overexpressed had intensities approximately three times greater than the average for normal controls and three samples in which p16 was two times love than the six hematogically normal control. In all cases the blots were reprobed with antibodies to actin to correct for loading differences (Figure 1, Table 1).

DISCUSSION

p16 which associates with CDK4 and inhibits catalytic activity of the CDK4/cyclin D complexes and cell proliferation[6-8]. Various frequency of p16 homozygous deletions have been reported by several authors in ALL Using Southern blot hybridisation. Takeuchi S analysed primer ALL samples and detected homozygous deletion of the gene in 77% T-ALL samples[4]. Several other groups also reported delated in of p16 in T-ALL although the frequency varies between 17-85%[13,14]. Here we detected p16 homozygous deletions in 19% in T-ALL. The rate in T-ALL is similar to the rates reported by other investigators.

Gene-promoter methylation is an epigenetic

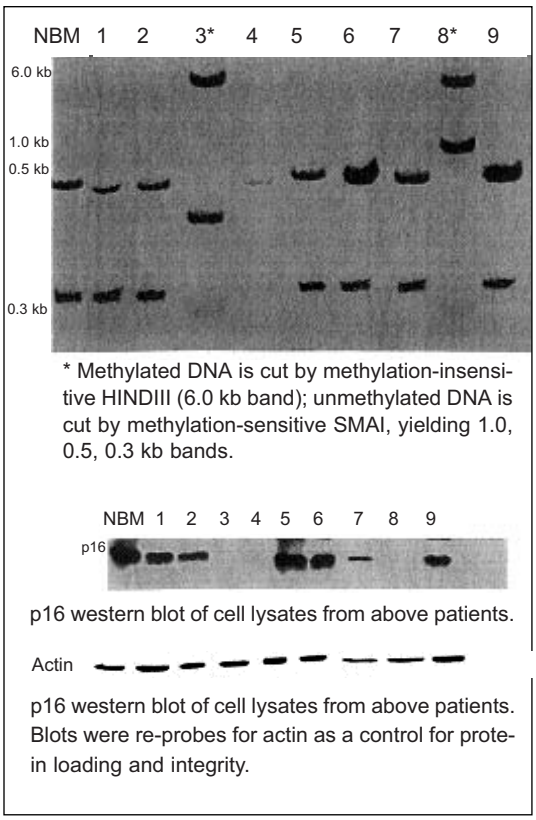

Figure 2. Southern and Western blot analyses of CDKN2 gene for methylation and expression in ALL patients.

a. Lane 1= genomic DNA from a normal bone marrow (NBM), lines1-9= patients were digested with HIND III and SMA I, electrophoresed, blotted and probed for p16 as described in materials and methods. Methylated DNA is cut by methylation-insensitive HIND III (6.0 kb band); unmethylated DNA is cut by metylation-sensitive SMA I, yielding 1.0, 0.5, 0.3 kb bands. Lane 3 and 8 methylated all the other lanes are unmethylated.

b. p16 Western blot of cell lysates from above patients.

c. Blots were reprobed actin as a control for protein loading and integrity.

mechanism of transcription inactivation. In this study, the authors investigated the frequency and prognostic significance of p16 gene methylation in ALL.

It was reported that methylation of the 5'-CpG island in exon 1 of the p16 gene silences its transcription in approximately 20% of different neoplasms and T-ALL in diagnosis 4/38 and relapse

Table 1. DNA methylation, deletion and p16 protein expression of p16 gene in ALL patients

Patient	Homozygous deletions	DNA methylation	p16 protein expression
P1	+	-	+
P2	+	-	+++
P3	del	-	-
P4	+	-	+
P5	+	-	-
P6	del	-	-
P7	+	+	-
P8	del	-	-
P9	+	-	++
P10	+	+	+++
P11	+	-	+++
P12	+	-	++
P13	+	+	-
P14	+	-	++
P15	+	-	++
P16	del	-	-
P17	+	-	+
P18	+	-	++
P19	+	-	-
C1	+ control	-	++
C2	+ control	-	++
C3	+ control	-	+++
C4	+ control	-	++
C5	+ control	-	++
C6	+ control	-	++

0/42[23]. Chim CS et al reported methylation of p16 gene in 6% in T-ALL. Delmer A et al. showed that p16 mRNA was detected in 5/17 ALL cases by RT PCR[24]. By Southern blotting, a homozygous deletion of p16 gene was found in 6/17 ALL cases among which 4/6 were negative or weakly positive by RT-PCR. In this study, we concentrate on DNA methylation in T-ALL and cell lines. Only three patients were methylated at the 5'CpG island using HIND III and SMA I enzyme but all others showed no methylation at all. And lack of p16 protein expression occur in 8/19 in T-ALL patients. These results explain that lack of p16 gene expression is very common in ALL because of deletion and mutation. Methylation of CpG island of p16 gene may not play a big role regarding p16 protein expression in T-ALL. This finding also shows that there is an alternative mechanism of p16 inactivation such as mutation, mainly by homozygous gene deletion[1,2,25]. In cell lines, before 5-AzoCyt treatment, RD cell line have not p16 protein expression, but using 5-AzoCyt the cell line showed p16 expression. This has not been noticed with the other cell lines The result show that; p16 gene expression in RD cell line may be controlled by DNA methylation.

Table 2. Homozygous deletion and p16 protein expresssion of p16 gene in cell lines

Cell lines		p16 protein expression	
	Homozygous p16 delation	Before 5-AzoCyt	After 5-AzoCyt
91.06 (EU-8	del	-	-
REH	del	-	-
855 (EU-1)	del	-	-
U937	+	+	+
93-27 (EU-12)	+	+++	+++
RD (Uoc B4)	+	-	++
93-10 (EU-9)	+	+	+
EI (Sup-B2)	+	+++	+++
AR (Uoc-B11)	+	+	+
697 (EU-3)	+	+	+
HI-60	+	-	-

HL-60 cell line studied, p16 protein expression was not absent in contrast to Otterson GA et al reported that HL-60 cell line have p16 protein expression[26]. This may be due to the secondary event arising during in vitro culture. The remaining cell lines expressed p16 at constant levels before and after 5-AzoCyt treatment.

These hints give us a reason to think about other possible and hence unknown mechanisms that controls p16 gene expression.

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