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Hypermethylation of CpG islands in the promoter region of the *p15INK4B* gene in childhood acute leukaemia

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Abstract

It has been reported that the cyclin-dependent kinase inhibitor (CDKI) gene *p15INK4B* is frequently inactivated by genetic alterations and may be responsible for various malignant tumours. Another way of inactivation of this CDKI is by hypermethylation of 5′CpG islands in the promoter region of the *p15INK4B* gene and this inactivation seems to be a frequent event in various haematological malignancies. In the present study, we investigated the methylation status of the *p151NK4B* gene to clarify its role in the pathogenesis of childhood acute myeloid (AML) and acute lymphoblastic leukaemia (ALL). The study included 23 cases of B-cell origin ALL, 13 cases of T-cell origin ALL, 32 cases of AML, and 10 apparently healthy controls. Hypermethylation was studied by methylation-specific polymerase chain reaction. Hypermethylation of the *p15INK4B* gene was more frequent in cases with T-cell origin ALL (46.2%), but similar among children with B-cell origin ALL (13.0%) and AML (18.8%). Hypermethylation of *p15INK4B* may be involved in the pathogenesis of T-cell origin ALL, but not in that of AML or B-cell origin ALL.

Keywords: Hypermethylation; CpG island; Cyclin-dependent kinase inhibitors; Acute myeloid; Acute lymphoblastic leukaemia

1. Introduction

Epigenetics is described as a heritable change in gene expression without an alteration in the coding sequence. The main epigenetic modification in mammals, and particularly in humans, is DNA methylation, which is catalysed by DNA methyltransferase and involves the addition of a methyl group to the carbon-5 position of the cytosine ring converting it to methyl cytosine [1].

The target sequence of cytosine methylation, the CpG dinucleotide, has a disproportional and non-random distribution through the human genome. The CpG dinucleotide has been progressively depleted during human evolution due to the high mutational rate of 5-methyl-cytosine, which is converted to thymine after spontaneous deamination; a phenomenon called CG

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suppression. Small areas (300 bp to several kb) that contain the expected frequency of CpGs (1/16), are called CpG islands. These areas are located at the 5′ end (promoter, untranslated region and exon 1) of approximately 60% of human genes [2] and they are protected from methylation in normal cells. This protection is probably due to the action of the protein Sp1 binding to sites in or near these regions of DNA and also through the action of embryo-specific factors. However, fully methylated CpG islands can be found only in the promoters of silenced alleles for selected imprinted genes and multiple silenced genes on the inactive X-chromosome of females [3]. In addition, DNA methylation is essential for normal mammalian development and embryogenesis, cellular differentiation, chromosome integrity, control of DNA replication and repair. Finally, DNA methylation is essential for the transcriptional repression of endogenous parasitic DNA as a host defence mechanism [1].

Methylation of promoter CpGs is associated with a closed chromatin structure and results in the transcriptional silencing of the associated gene that is clonally propagated through mitosis by the action of DNA methyltransferase enzymes [4]. Aberrant methylation patterns are associated with some developmental diseases, aging and cancer. Specifically, aberrant promoter hypermethylation of selected genes that involve cell cycle control, DNA repair, cellular differentiation, apoptosis, metastasis and resistance to drugs is associated with transcriptional silencing of flanking genes. This constitutes an epigenetic mechanism of loss of gene function in cancer that is alternative to mechanisms involving genetic lesions [4,5].

The regulation of the cell cycle is under the control of some important regulator molecules, such as the products of tumour suppressor genes (TSGs), p53 and pRb. Positive and negative regulators contribute to the normal function of the cell cycle [6]. Among the negative regulators, *p15INK4B* and *p16INK4A*, two neighbouring genes at chromosome 9p21 that are functional homologues, are considered to be TSGs, according to their mode of action. The protein products of *p15INK4B* and *p16INK4A* are cyclin-dependent kinase inhibitors (CDKI) because they share similar functional domains (ankyrin repeats for protein–protein interactions), which enable them to compete with cyclin D for CDK4 and CDK6 [7]. As a result of the inhibition of the cyclin D/CDK4 complex, RB remains in its active hypophosphorylated form and can complex with transcription factor(s) (E2F family) of critical genes allowing cells to progress to the S1 phase of the cell cycle. E2F becomes inactive and the cell is arrested in the G1 phase of the cell cycle. Altered cell cycle control underlies the development of many human neoplasias. Normal expression of *p15INK4B* mRNA in haematopoietic cells is relatively low compared with that in other tissues, such as testis [8]. Stone and colleagues found that in stimulated T lymphocytes, levels of *p15INK4B* mRNA remain unchanged throughout the cell cycle, irrespective of the pRb status, suggesting that the *p15INK4B* gene does not regulate the cell cycle in mature T cells [9]. Erickson and colleagues showed that *p15INK4B* and *p16INK4A* proteins are expressed in primary T lymphocytes and maintain cell quiescence, and these genes, as well as the RB1 gene are important for T-cell senescence [10]. Their contribution to the malignant transformation of cells may be through alteration of cell senescence.

In many cancers, the *p16INK4A* and *p15INK4B* genes are inactivated by homozygous deletions [11] and also by promoter hypermethylation. *P16INK4A* is hypermethylated in many tumour types, including bladder, cervical tumours or melanomas, gliomas, colorectal, lung, breast and head and neck carcinomas [12]. Hypermethylation of *p15INK4B* is only observed in haematological malignancies [13,14] as *p15INK4B* expression is activated by transforming growth factor β (TGF-β), which is expressed mainly in haematopoietic cells and therefore epigenetic inactivation of *p15INK4B* offers a survival advantage to these cells.

In haematological malignancies, *p16INK4A* *p15INK4B* deletions occur frequently in acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma patients, but in myeloid malignancies, the frequency of homozygous deletions appears to be very low suggesting other mechanisms are involved [15]. *P15INK4B* hypermethylation is frequently observed in Myelodysplastic Syndrome (MDS), acute myeloid leukaemia (AML) and ALL, but this is not the case for the *p16INK4A* gene [16,17]. Variations in the methylation of these genes are observed in studies including adult and childhood leukaemias [17–19].

Hypermethylation of the *p15INK4B* promoter may play an important role in the pathogenesis of leukaemias. This epigenetic change is an attractive target for therapeutic intervention as it is reversible after treatment with demethylating agents (DNA methyltransferase inhibitors) such as 5′ deoxy-azacytidine [20]. In this study, we investigated the frequency of *p15INK4B* promoter hypermethylation in childhood acute leukaemias. We examined the methylation status of the *p15INK4B* promoter in 78 children to investigate whether hypermethylation had a differential role in different types of leukaemia and/or correlated with the age and/or gender of the patient.

2. Patients and methods

2.1. Patients and DNA

The six Paedriatric Hematology–Oncology Departments operating in Greece and the Department of

Hygiene and Epidemiology of the Athens Medical School have established a nationwide network to undertake epidemiological research concerning childhood leukaemia [21]. During a seven-year period (1996–2002), a total of 584 cases were diagnosed and sufficient quantities of blood samples were available for 432 cases at the beginning of 2003 for our study. We randomly selected two thirds (35) of the 49 available cases of AML and ten percent (40) of the 383 available cases of ALL. The unequal sampling ratios were chosen to increase the statistical power of the study. We have also randomly selected 10 controls from a large pool of children with minor conditions who had blood taken for diagnostic purposes and had been used in several previous case-control investigations [22,23]. From the blood samples of the 85 children, seven (3 AML and 4 ALL) could not be used for technical reasons. Informed consent from the parents of the children and blood samples was obtained earlier for routine procedures. The present study was approved by the University of Athens Medical School Ethics Committee.

Blood samples were obtained by venipuncture before the initiation of any treatment; thereafter, peripheral-blood mononuclear cells (PBMC) were isolated from the specimens and cryo-preserved within 6 h of the time of collection, according to the protocol followed by the National Retroviruses Reference Center of Greece. Genomic DNA was extracted from all samples at the National Retroviruses Reference Center, which is based at the Department of Hygiene and Epidemiology in Athens Medical School [24].

2.1.1. Methylation-specific polymerase chain reaction

Analysis of the methylation status of the promoter regions of the *p15INK4B* gene was performed by methylation-specific polymerase chain reaction (MSP), as described elsewhere in [25]. This method is specific and sensitive for the study of methylation of CpG sites in a CpG island. The method consists of two steps: (a) modification of DNA using sodium bisulphite, which converts all unmethylated, but not methylated cytosines to uracil and (b) amplification of the bisulphite-modified DNA by the polymerase chain reaction (PCR) using specific primers for methylated versus unmethylated DNA.

2.1.1.1. Bisulphite modification. DNA was treated with sodium bisulphite for conversion of unmethylated cytosines to uracils, leaving unaffected the methylated cytosines, as described previously in [25].

2.1.1.2. PCR amplification. Bisulphite-modified DNA was amplified by PCR using the primer sets described in Table 1. Primers were purchased from Minotec (Crete). Universal methylated genomic DNA and unmethylated genomic DNA (Intergene USA), after chemical modification, were used as positive and negative controls in each experiment.

Unmodified (wild) DNA was amplified with a wild primer set to serve as a positive control for the PCR and DNA quality and a negative PCR control (with no DNA template) was performed for each set of primers to serve as controls for contamination. Amplification was carried out over 35 cycles (denaturation for 30 s at 95 °C, annealing for 30 s at the specific temperatures listed in Table 1, and extension for 30 s at 72 °C). An initial denaturation step (3 min at 95 °C) and a final extension step (5 min at 72 °C) were also performed.

The generated fragments were subjected to electrophoresis on non-denaturing 10% polyacrylamide gels, stained with ethidium bromide and directly visualised under ultraviolet (UV) illumination.

2.2. Statistics

For the analysis, data were initially classified by immunophenotypic diagnostic category and the hypermethylation status. Subsequently, the odds for hypermethylation were compared among the various diagnostic categories using multiple logistic regression, and also controlling for age and gender. We have used as a reference group, the B-cell origin ALL, which is the most common category of childhood leukaemia. We did not use as reference group healthy controls because their limited number would not allow precise estimates to be calculated and the focus of our study was on the possible differential role of hypermethylation in the different leukaemia groups.

3. Results

3.1. p15INK4B promoter hypermethylation in childhood leukaemia

Results obtained from the analysis of the methylated status of CpG islands in the promoter region of the

Table 1
Primer sequences for the methylated (p15M), unmethylated (p15U) and wild-unmodified promoter (p15W) of *p15INK4B* gene

	Sense primer 5′ 3′	Antisense primer 5′ 3′	Size (bp)	Annealing temperature (°C)
P15 W	Cgcaccctgcggccaga	agtggccgagcggccgg	137	65
P15 M	Gcgttcgtattttgcggtt	cgtacaataaccgaacgaccga	148	60
P15 U	attttgttagagtgaggtggg	tccaaacttttcctaacactc	157	60

The size of the expected fragments and the annealing temperatures used in these reactions are also presented.

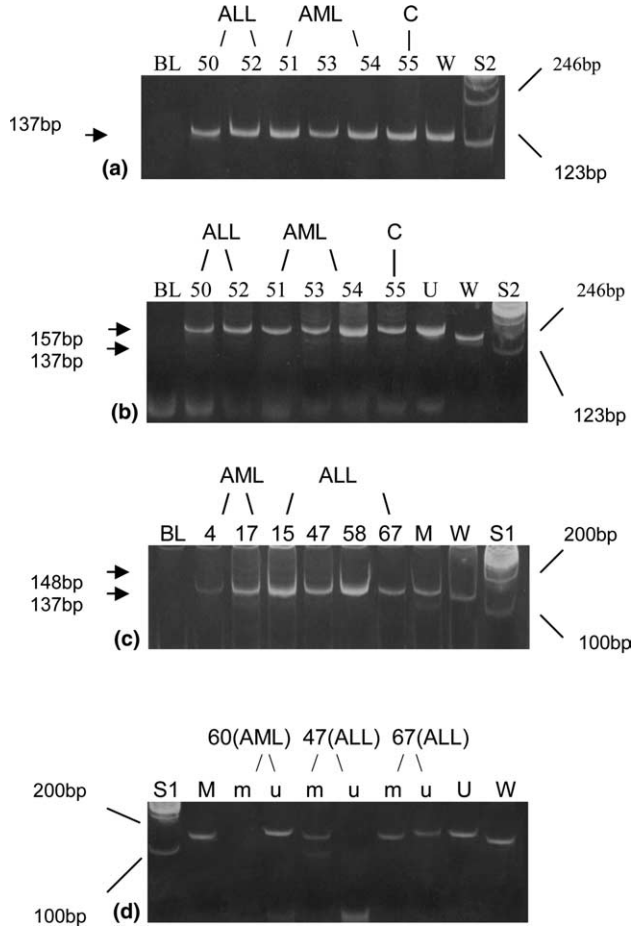

Fig. 1. MSP *p15INK4B* promoter region in childhood patients with AML and ALL. Three different PCR reactions were carried out: one with unmodified DNA and wild-type primers (W) served as a positive control for PCR and DNA quality (a) and two reactions with bisulphite-modified DNA and primers specific for the unmethylated (b) and methylated sequence (c). Figure (d) shows same sample of bisulphite-treated DNA with both methylated (m) and unmethylated (u) primers. Bisulphite modified DNAs containing fully methylated DNA (M), fully unmethylated DNA (U) and distilled water were used respectively as positive and negative control of methylation and as control of contamination in each reaction. S1, size molecular marker (100 bp ladder DNA); S2, size molecular marker (123 bp ladder DNA); BL, PCR reaction containing distilled water instead of DNA; C, DNA extracted from control, normal DNA.

p15INK4B gene in paediatric patients with AML and ALL are presented in Fig. 1 and summarised in Table 2. The PCR with unmodified DNA and wild primers yielded a 137 bp band, whereas bisulphite-modified DNA with primers specific for methylated and unmethylated DNA yielded bands of 148 and 157 bp, respectively (Fig. 1).

p15INK4B showed methylation in a substantial proportion of T-cell origin ALL cases (46.2%), whereas the proportion of cases with *p15INK4B* hypermethylation appears similar among controls (20.0%) and among children with either B-cell origin ALL (13.0%) or AML (18.8%) (Table 2).

3.2. *p15INK4B promoter hypermethylation according to the demographic characteristics of the patients and leukaemia type*

Table 2 shows the age, gender and hypermethylation status in the four groups studied. There is no evidence for a significant difference according to the age or gender distribution among the groups ($P > 0.20$ and 0.15, respectively). Moreover, the proportion of cases with hypermethylation appears similar among controls and among children with either B-cell origin ALL or AML. In contrast, hypermethylation was apparently more frequent among cases with T-cell origin ALL. However, some confounding by age and gender cannot confidently be excluded, even though the four groups were similar with respect to these variables.

Table 3 shows multiple logistic regression-derived Odds Ratios and 95% Confidence Intervals for hypermethylation for each study group. The odds for hypermethylation is five times higher in the DNA from the white blood cells of children with T-cell origin ALL than in the other three groups of B-cell origin ALL, AML, and controls (MODEL 1). There is no significant evidence for a variation in the frequency of hypermethylation in the other three groups (MODEL 1). The significant excess of hypermethylation among children with T-cell origin ALL persists, even after adjustment for age and gender (MODEL 2).

Table 2
Age, gender and hypermethylation status in the four groups

Study Group	*N*	Age* (years)		Percent of boys**	Hypermethylation yes (%)
		Mean	SD		
Controls	10	5.6	4.8	50.0	20.0
AML	32	6.6	5.4	50.0	18.8
B-cell ALL	23	4.5	3.3	56.5	13.0
T-cell ALL	13	6.2	4.4	84.6	46.2

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; SD, standard deviation.
* Differences by age were statistically non-significant (*P*-value derived from ANOVA > 0.20).
** Differences by gender were statistically non-significant (*P*-value derived from χ^2 test >0.15).

Table 3
Multiple logistic regression-derived Odds Ratios (ORs) and 95% Confidence Intervals (95% CIs) for hypermethylation for each study group

Variable	Category or increment	ORs	(95% CIs)	*P* value
MODEL 1				
Study group	Controls	1.67	(0.23–11.93)	0.61
	AML	1.54	(0.34–6.92)	0.57
	B-cell ALL	Baseline		
	T-cell ALL	5.71	(1.12–29.21)	0.04
MODEL 2[a]				
Study group	Controls	1.74	(0.24–12.83)	0.59
	AML	1.62	(0.34–7.70)	0.54
	B-cell ALL	Baseline		
	T-cell ALL	5.22	(1.00–27.70)	0.05

[a] Controlling for age and gender.

4. Discussion

We investigated the frequency of *p15INK4B* promoter hypermethylation among paedriatric patients with B-cell origin ALL, T-cell origin ALL and AML. A significantly higher proportion of T-cell origin ALL cases were characterised by *p15INK4B* promoter hypermethylation (46.2%) compared with apparently healthy children and children with B-cell origin ALL and AML (20.0%, 13.0% and 18.8%, respectively). Hypermethylation of *p15INK4B* (alone or in combination with *p16INK4A* deletion or hypermethylation of other critical genes) in cases of paedriatric ALL has been found in previous studies [19,26,27], but in some investigations a higher prevalence of *p15INK4B* promoter hypermethylation has been reported for AML rather than ALL [17,18,28]. Thus, the significance of *p15INK4B* epigenetic inactivation in specific lineages remains unclear.

In this study, we were unable to establish a direct correlation of methylation with gene expression due to the lack of sufficient RNA. Previous studies have shown that for *p15INK4B*, methylation is associated with gene silencing [26].

MSP analysis was also carried out using the peripheral blood from 10 normal children. Two of 10 children were positive for methylation and this finding was a not-expected one. Since we excluded the possibility of a non-specific amplification, this indicates that a small fraction of normal cells or alleles are methylated in the promoter of the *p15INK4B* gene. MSP is a highly sensitive technique and can detect one methylated allele in 1000 unmethylated alleles [25].

A similar finding has been reported by other investigators [29]. They separated mono nuclear cells (MNCs) from peripheral blood into lymphocytes and monocytes by immunomagnetic bead isolation. The results from the MSP analyses of bisulphite-treated DNA showed that the lymphocyte fraction was positive for methylation, whereas the monocyte fraction was negative. The biological significance of *p15INK4B* methylation in a subpopulation of normal lymphocytes remains unclear. It is possible that hypermethylation of some CpGs in a gene region may not correlate with transcriptional silencing. This phenomenon has been documented in studies concerning hypermethylation of the *p16INK4A* gene in normal breast tissue and normal colon mucosa and hypermethylation of *p15INK4B* gene in normal colon mucosa [30]. Cancer-related genes that show some degree of methylation in normal haematopoietic cells include p21, IGF2 and *p15INK4B* [31]. It seems that the density of critically located methylated CpGs within a CpG island, and not any specific location, correlates best with transcriptional loss, so that a few methylated CpGs can silence a weak promoter, whereas a higher density of methylation is required to repress a strong promoter.

The major difference between silencing through gene mutations and epigenetic gene silencing is that the latter is potentially reversible via the action of demethylating agents (DNA methyltransferase inhibitors), such as 5′ azacytidine and 5′ deoxy-azacytidine [32]. This offers the opportunity to develop agents that manipulate this pathogenetic pathway. Indeed, 5′ azacytidine, known for 20 years as an antineoplastic agent, is of clinical use, mainly against haematopoietic neoplasms as it acts as demethylating agent allowing re-expression of the silenced genes [33]. However, it is worth noting that administration of 5′ azacytidine must be continuous in order to maintain the inhibition of DNA methylation. Thus, if our results were confirmed they would provide a diagnostic and prognostic tool for T-cell origin ALL and they might even contribute to the formulation of a potentially useful treatment indication.

Our findings indicate a high frequency of *p15INK4B* methylation in childhood T-cell origin ALL that could be a useful biomarker for malignant transformation and prognosis. Since this epigenetic modification can occur early during oncogenesis [13] and the positive PCR signal cannot be masked by the presence of normal cells, the biomarker could contribute to the early detection and assessment of minimal residual disease among children with T-cell origin ALL. However, appropriate quantitative assays need to be established, because we found some methylation in the blood cells of healthy children.

Conflict of interest statement

All authors of this research study disclose that they do not have any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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