Down-regulation of *DLX3* expression in *MLL-AF4* childhood lymphoblastic leukemias is mediated by promoter region hypermethylation

MARTA CAMPO DELL'ORTO[1], BARBARA BANELLI[2], EMANUELA GIARIN[1], BENEDETTA ACCORDI[1], LUCA TRENTIN[1], MASSIMO ROMANI[2], GEERTRUY TE KRONNIE[1] and GIUSEPPE BASSO[1]

[1]Department of Paediatrics, University of Padova, via Giustiniani 3, I-35128 Padova; [2]Laboratory of Experimetal Oncology 'C', Largo Rosanna Benzi 10, I-16132 Genova, Italy

Received February 2, 2007; Accepted April 14, 2007

Abstract. Hypermethylation of CpG islands is the most well defined epigenetic change in neoplasia and plays an important role in the inactivation or silencing of cancer related genes. *DLX* genes (1-7), with large CpG islands in their 5' region, are implicated in a number of processes among which haematopoiesis. They are characterized by highly dynamic spatio-temporal expression and supposed to be involved in resistance to apoptosis of several tumor cell lines. In acute lymphoblastic leukemia (ALL) hypermethylation is a common phenomenon frequently associated with poor prognosis in specific genetic childhood leukemia subgroups. These data together with the presence of large CpG islands in the up-stream regions of the *DLX* genes make them attractive candidates for methylation regulated gene expression and leukemia related aberrancies. To validate the role of *DLX* genes in paediatric B-ALL cells, we studied two cell lines and two groups of patients with paediatric chromosomal rearrangements: *MLL-AF4* and *TEL-AML1*, respectively. Analysis of methylation and gene expression patterns of *DLX3* in 64 specimens of B-lineage ALL revealed that *DLX3* presents aberrant methylation in paediatric B-ALL patients. *In vitro* experiments with 5-Aza-2'dC on leukemia cell lines, confirmed by Western blot analysis, indicated that the methylation of *DLX3* CpG islands has a functional role and interferes with the *DLX3* gene and DLX3 protein expression in B-ALL cells. Importantly, hypermethylation of *DLX3* significantly reduces its expression in *MLL-AF4* rearranged leukemias while methylation is almost absent in *TEL-AML1* positive ALL specimens. These results show that differential *DLX3* methylation could be a new epigenetic marker for genotypic B-cell leukemia subgroup with high-risk features.

Correspondence to: Dr Geertruy te Kronnie, Department of Paediatrics, University of Padua, via Giustiniani 3, I-35128 Padua, Italy
E-mail: truustekronnie@unipd.it

Key words: methylation, *DLX*, acute paediatric leukemia, 5-Aza-2'dC

Introduction

Chromosomal translocations that inactivate or create new fusion genes are a common hallmark of acute lymphoblastic leukemia (ALL) and may serve as diagnostic and prognostic markers in subtypes of ALL (1,2). However, translocations alone do not result in leukemeogenesis; other aberrancies that are either the consequence of the translocation or an independent secondary event have to occur before leukemia develops (3). It has been demonstrated that aberrant methylation of clustered cytosine-guanosine motifs (CpG), especially in CpG islands located in gene promoter regions, is an early and essential step in tumor development and methylation has been proved to be a mechanism of gene silencing as common as the disruption of tumor-suppressor genes by mutation or deletion (4-6). Moreover, DNA hypermethylation and transcriptional silencing of genes involved in tumor invasiveness, cell growth and apoptosis, may also influence recurrence after treatment and overall survival. Inactivation of cancer-related genes by DNA methylation is a frequent event also in paediatric and adult ALL and, by looking at differences in the methylation pattern of multiple genes, specific risk groups among patients have been identified (7). It is understood that each type of cancer may have several genes susceptible to promoter hypermethylation and individual tumors would exhibit different frequencies of hypermethylation patterns of multiple genes, eventually related to patient's clinical outcome (8,9). No systematic identification of aberrant genomic methylation of neoplasias or leukemias has so far been performed while several studies have focused on silencing of specific genes in specific subtypes of leukemias (10,11). The identification of novel DNA methylation-based prognostic indicators requires the selection of suitable candidate genes.

The *DLX* genes (*DLX1-7*) implicated in haematopoiesis and in a number of other processes with highly dynamic spatio-temporal expression patterns (12) and well-defined CpG islands in their promoter region, are attractive targets for methylation studies in leukemia subtypes. In particular, the

down-regulation of *DLX2*, *3* and *4* has been described in a group of paediatric B-ALL characterized by the t(4;11) (*MLL-AF4*) chromosomal rearrangement (13). Furthermore, it has been reported that this down-regulation is directly connected to an increased resistance to apoptosis in several cancer cell lines (14). The presence of extended CpG islands at the 5'-end of *DLX2*, *3* and *4* genes, their possible role in resistance to apoptosis, and the recent finding of *DLX5* promoter methylation as one of the epigenetic markers of chronic lymphoblastic leukemia (CLL) (15), prompted us to study the methylation and gene expression pattern of *DLX2*, *3* and *4* in specific paediatric ALL subtypes.

Among the paediatric ALL, there are two fundamental subtypes: the first is that of B-ALL carrying the t(4;11) (q21;q23)(*MLL-AF4*) rearrangement present in the 3% of paediatric leukemia and in 70% of infants (16); the second is represented by B-ALL carrying the t(12;21) (p13;q22)(*TEL-AML1*) chromosomal aberration that, rare in adult B-ALL, is the most frequent (25-30%) in childhood lymphoblastic leukemia (17-19).

We analyzed the methylation status of *DLX* genes in cell lines representative of specific leukemic sub-types and the effect of demethylation on their gene expression. The remarkable *DLX3* methylation-dependent inactivation, supported by reduced DLX3 protein expression, prompted our study on the *DLX3* gene. To determine if the methylation status of *DLX3* could identify two leukemia sub-types, we analyzed *DLX3* CpG island methylation and gene expression in a group of paediatric B-ALL characterized by *MLL-AF4* chromosomal rearrangement, and compared these with *TEL-AML1* positive childhood patients; besides we used a more heterogeneous population of B-ALL without the above-mentioned chromosomal alterations, as control group. Demonstrating that *DLX3* methylation has a functional role in regulation of the *DLX3* expression, *DLX3* silencing in RS4;11 cell lines have been reversed treating cells with DNMT1 (DNA methyltransferase 1) inhibitor. Down-regulation of *DLX3* expression in relation to methylation has also been confirmed in paediatric B-ALL where *DLX3* is significantly down-regulated in *DLX3* promoter methylated specimens. Further-more we showed that *DLX3* has the highest percentage of methylation and the lowest gene expression in *MLL-AF4* positive B-ALL when compared to *TEL-AML1* and control not-translocated specimens.

Materials and methods

Leukemic and normal cell lines. The following leukemic cell lines were used: HL60 (human acute myeloid leukemia), REH [B-ALL with t(12;21)(*TEL-AML1*)], RS4;11 [human B cell precursor leukemia harbouring the t(4;11)(*MLL-AF4*)], U937 [human histiocytic lymphoma with t(10;11)(*MLL-AF10*)]. REH and RS4;11 cell lines were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmgH, Braunschweig, Germany). U937 cell line, chosen as an example of *MLL* translocation in myeloid leukemic cells, was obtained from the Cell Lines Bank of the IST, Genova, Italy. As normal cell line control, we used the LB1 cell line derived by EBV immortalization from B cells of a healthy donor. Cell lines were maintained in RPMI-1640 (Biochrom AG, Germany) 10% FBS, incubated at 37˚C and 5% CO_2 and with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Leukemic and normal samples. The clinical samples utilized in our study were bone marrow aspirates (BM) or peripheral blood (PB) lymphocytes collected from 64 paediatric B-ALL patients from the Department of Paediatrics of Padua. This study was conducted after obtaining the informed consent from all patients following the tenets of the Declaration of Helsinki and was approved by the ethics committees of the participating institutions before the initiation of the study. Out of these, 34 were t(4;11)(q21;q23)(*MLL-AF4*) leukemias; 17 were t(12;21)(p13;q22)(*TEL-AML1*) positive. The remaining 13 samples lacked t(4;11) and t(12;21) translocations; moreover they lacked the other rearrangements t(1;19)(q23;p13)(*E2A-PBX1*) and t(9;22)(q34;q11)(*BCR-ABL*) routinely screened for the diagnosis of paediatric B-ALL. In all cases the percentage of blast cells ranged from 60 to 97%. Molecular diagnosis of t(4;11) and t(12;21) aberrations were accomplished following protocols defined by BIOMED-1 Concerted Action (20). All patients were risk-stratified according to the therapeutic protocol based on standard prognostic features. As normal control samples we utilized a pool of five PB lymphocytes (PBL), a healthy BM aspirate and $CD19^+$ cells isolated from a healthy donor as representative of normal B cells. Mononuclear cells from patients, PB and healthy BM were isolated using Ficoll-Hypaque (Pharmacia-LKB, Uppsala, Sweden) density gradient centrifugation. $CD19^+$ B cells were separated from control PB with CD19 micro-beads (Miltenyi Biotec Inc., USA) according to manufacturer's instructions; the purity was consistently over 95%, as determined by flow cytometry analysis.

Treatment of leukemia cell lines with 5-Aza-2'dC. To achieve demethylation, RS4;11 and REH cell lines were treated with 5-Aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 5 μM for 72 h. The 5-Aza-2'-deoxycytidine (5-Aza-2'-dC) containing culture medium was refreshed daily. Treated cells were harvested after 24, 48 and 72 h of incubation to extract DNA for methylation-specific PCR (MSP) analysis, total-RNA for real-time RQ-PCR and proteins for Western blot (WB) analysis as described below.

DNA and RNA extraction, and cDNA synthesis. Genomic DNA and total-RNA from cell lines and patients were extracted using a Purogene Kit (Gentra, MN, USA) and TRIzol reagent (Invitrogen, Karlsruhe, Germany) respectively. Total-RNA quality was verified using the Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano Assay kit (Agilent Technologies, Waldbronn, Germany). RNA was then reverse-transcribed: 1 μg of total-RNA was incubated with random primers (Invitrogen) for 10 min at 70˚C and subsequently subjected to reverse transcription for 10 min at 25˚C and 30 min at 42˚C, followed by heating at 99.9˚C for 5 min. Reactions were performed in a final volume of 20 μl.

Sodium bisulfite modification of DNA and methylation-specific polymerase chain reaction (MSP). Methylation analysis was carried out by methylation specific PCR (MSP)

Table I. *DLX2*, *DLX3* and *DLX4* primer sequences used for MSP analysis.

Gene	Accession no. and locus	MSP primers	Annealing temperature	MSP product
DLX-2 M	NM_004405 2q32	Fw: tgatttttcgttatttcgatttttc Rv: acactcgtcacgtaaacgctaa	56	206
DLX-2 U		Fw: ttttgatttttttgttattttgatttttt Rv: catttaacactcatcacataaacactaa	52	215
DLX-3 M	NM_005220 17q21	Fw: tttgacggtaggtaatggtgtaagc Rv: acgaaacaaaaatatatatccaaaaaacga	58	100
DLX-3 U		Fw: ttgatggtaggtaatggtgtaagtgt Rv: aaaacaaaaatatatatccaaaaaacaaa	56	97
DLX-4 M	NM_138281 17q21.33	Fw: ttttagatttcgtttttgtttcgtc Rv: ctccgaaattcgctaactcg	58	147
DLX-4 U		Fw: ttttagattttgtttttgttttgttg Rv: ataaaaatctccaaaattcactaactca	56	155

Fw, forward primer; Rv, reverse primer, M, primers for the methylated allele; U, primers for the unmethylated allele. Annealling temperature (˚C) and each MSP product length (bp) are also reported.

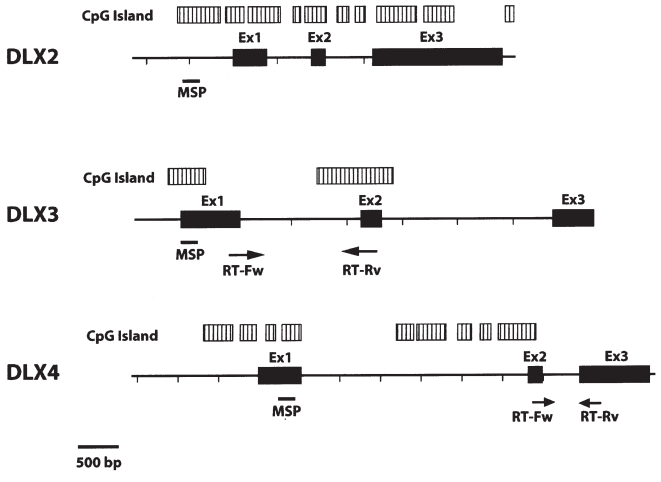

Figure 1. Genomic organization of *DLX2*, *DLX3* and *DLX4* loci. MSP, sites at the 5'-end of *DLX2*, *3* and *4* assayed for their methylation status. ex, exons. RT-Fw (forward) and RT-Rv (reverse), real-time PCR primers. CpG island, localization of CpG islands along each gene.

as previously described (21). Briefly, DNA was treated with sodium bisulfite for 16 h at 50˚C to convert the unmethylated cytosines (C) to uracil leaving the methylated C unmodified. After purification the DNA was subjected to DNA amplification with primer sets recognizing solely the methylated or the unmethylated target and the PCR products were resolved on a 3% agarose 1000 gel (Invitrogen). The presence of amplification bands only with one set of primers indicates complete methylation (or unmethylation), whereas the amplification with both sets of primers is indicative of partial methylation (hemi-methylation).

Bisulfite modified DNA was used as template for MSP to analyze the methylation pattern of *DLX2*, *DLX3* and *DLX4* 5' regions with specific primers (Table I). The availability of DNA allowed the MSP analysis on 59 of the 64 patients included in this study. Primers were designed in the CpG-rich regions -1000 bp to 500 bp relative to the beginning of the genes (Fig. 1).

Exon/intron organization was deduced by aligning the reference sequences NM_004405 (*DLX2*), NM_005220 (*DLX3*) and NM_138281 (*DLX4*) on the human genome sequence. The transcription start of *DLX3* was deduced by homology searching with the murine mRNA reference sequence for *DLX3* (NM_010055). The start of transcription of the *DLX4* gene has been independently identified (22). CpG islands were located with the CPGPLOT software (http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot.html). All MSP analyses were performed using CpGenome Universal Methylated DNA (UMD) (Chemicon, Temecula, CA) as positive control for methylated alleles and bone marrow (BM) from a normal donor as positive control for unmethylated alleles.

Real-time quantitative analysis. The mRNA expression levels of *DLX3* and *DLX4* were quantified by SYBR Green real-time quantitative PCR (RQ-PCR), using specific primers (Table II and Fig. 1) and the housekeeping gene *Gus-B*, glucuronidase-ß, as reference. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). RQ primers for *DLX4* were designed to detect all known splicing variants of *DLX4*. Reactions were performed in a final volume of 25 μl, containing 2 μl of cDNA, 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG 2X (Invitrogen, Life Technologies Inc., CA), and 0.5 μl of ROX reference dye. Reactions were performed on ABI PRISM 7700 SDS

Table II. *DLX3*, *DLX4* and *GUSB* primer sequences and quantity used for SYBR green RQ-PCR analysis.

Gene	SYBR green real-time primers	Quantity used in reaction mixture (nM)
DLX3	Fw: ttactcgcccaagtcggaatata	300
	Rv: ttcaccatgcgcacctctg	300
DLX4	Fw: cagactcggagaagccgc	300
	Rv: gaattctgcttcaggagcttcttatac	300
GUSB	Fw: gaaaatatgtggttggagagctcatt	50
	Rv: cggagtgaagatccccttttta	50

Fw, forward primer; Rv, reverse primer.

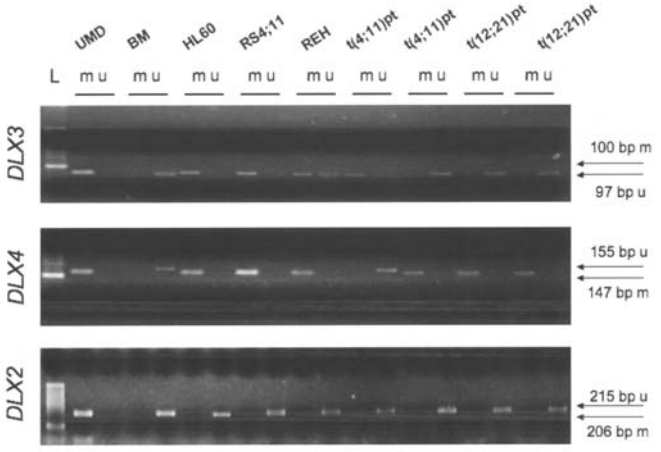

Figure 2. MSP results for *DLX3*, *DLX4* and *DLX2*. L, 25 bp DNA marker. UMD, CpGenome universal methylated DNA used as positive control for methylated alleles; BM, normal bone marrow used as positive control for unmethylated alleles; HL60, human acute myeloid leukemia cell line. RS4;11, human leukemia B cell line with t(4;11)(q21;q23)(*MLL-AF4*); REH, human leukemia B cell line with t(12;21)(p13;q22)(*TEL-AML1*); t(4;11)pt, paediatric B-ALL at diagnosis with t(4;11)(*MLL-AF4*) translocation; t(12;21)pt, paediatric B-ALL at diagnosis with t(12;21)(*TEL-AML1*) translocation. 100 bp and 97 bp, methylated and unmethylated alleles of *DLX3*, respectively. 147 bp and 155 bp, methylated and unmethylated alleles of *DLX4*, respectively. 206 bp and 215 bp, methylated and unmethylated alleles of *DLX2*, respectively; m, methylated; u, unmethylated.

(Applied Biosystems). Thermal cycler conditions were: a first hold of 2 min at 50˚C, followed by a denaturising step of 2 min at 90˚C and then by 50 cycles at 95˚C (15 sec for cycle) and 60˚C (30 sec for cycle). To exclude the contamination of unspecific PCR products such as primer dimers, melting curve analysis was applied to final PCR products after the cycling protocol. The expression levels of the target genes *DLX3* and *DLX4* and the reference gene *Gus-B* were determined from the calibration curve using the following equation: Amount=10^((C_t-intercept)/slope). The target gene amount was then divided by the reference gene amount to obtain a normalized target value. The normalized gene target expressions were also calculated for un-treated RS4;11 cells lines (calibrator). Each of the cell-line/specimen-normalized gene target value was then divided by the calibrator-normalized gene target value to generate the final relative expression.

Protein extraction and Western blot analysis. Cells were solubilized in Cell Extraction Buffer (BioSource International, Inc., USA) (10 mM Tris, pH 7.4, 0.1% SDS, 10% glycerol) supplemented with Protease Inhibitor cocktail and phosphatase inhibitor cocktail both at the final concentration of 1 mM (Sigma-Aldrich). Protein concentrations were measured with the BCA protein Assay Kit (Pierce, Rockford, USA). Total protein (15 μg) was separated by SDS-PAGE (12%) and then transferred onto PVDF membranes. Membranes were quenced in Blotto A buffer (Santa Cruz Biotechnology Inc., CA, USA) and then incubated with specific primary antibody (Santa Cruz Biotechnology Inc.) for DLX3 protein (1:100). The blots were washed in TBST buffer (Tween-20 0.05%) (Santa Cruz Biotechnology Inc.) then incubated with horseradish peroxidase-conjugated secondary antibody (HRP) (1:2000) (Santa Cruz Biotechnology Inc.). The Enhanced chemiluminescence (ECL) Kit (GE Healthcare, UK) was used to visualize immunoreactivity. ß-actin was used as housekeeping control. Gels were imaged using a high-resolution scanner (Arcus II, Agfa). The BAP (Brightness Area Product) of the DLX3 bands was determined with a constant threshold after black/white inversion using the Adobe Photoshop 7 software (Adobe) (23). The BAPs of DLX3 were plotted versus the known amount of DLX3 loaded and a standard curve was built. DLX3 BAP was normalized on the corresponding BAP value of the housekeeping protein ß-actin.

Statistical analysis. *DLX3* gene expression levels for all patients were first compared considering *DLX3* methylation status among them and applying the Dunn's Multiple comparison test to compare variances. Besides, *DLX3* gene expression was compared among patients belonging to different leukemia genetic subtypes and applying the non-parametric Mann-Whitney t test.

Results

MSP and SYBR green RQ-PCR in normal controls and acute leukemia (AL) cell lines. *DLX3* is methylated in cell lines but completely unmethylated in normal control samples (Fig. 2 and Table III). SYBR Green real-time quantitative PCR on RS4;11 and REH cell lines upon treatment with the DNMT1 inhibitor 5-Aza-2'-dC, shows that *DLX4*, though methylated in cell lines but unmethylated in normal control samples (Fig. 2), does not achieve a considerable increase of gene expression during demethylation (data not shown). Moreover, *DLX2* not methylated in either AL cell lines and non-tumorigenic controls (Fig. 2), is not considered as target for subsequent gene expression studies. Importantly and conversely to *DLX2* and *DLX4* genes, *DLX3* expression is absent in untreated cell lines and dramatically increases after demethylation (2.00-E+06 in RS4;11 and 4.00-E+05 in REH cells - both after 72 h) (Fig. 3).

MSP and SYBR green RQ-PCR in paediatric B-ALL. We have analyzed the 5' sequence methylation status of *DLX3* and the

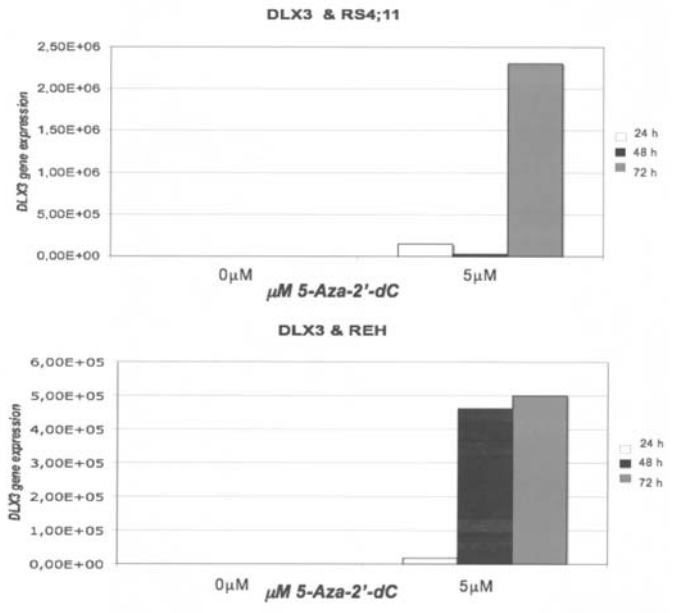

Figure 3. Increase of *DLX3* gene expression in RS4;11 (upper graph) and REH (lower graph) cell lines during treatment with 5-Aza-2'-deoxycytidine 5 μM. The 5-Aza-2'-deoxycytidine (5-Aza-2'-dC) containing culture medium was refreshed daily and treated cells were harvested after 24, 48 and 72 h of incubation to collect DNA, RNA and proteins. Gene expression of not treated cell lines (0 μM) was used as control.

Table III. Summary of samples used and corresponding MSP results.

Samples	No.	*DLX3 M*	*DLX3 U*
Cell lines			
RS4;11		+	-
REH		+	+
HL60		+	-
U937		+	-
Patients			
MLL-AF4/t(4;11)	34	24	10
TEL-AML1/t(12;21)	12	1	11
Not translocated	13	5	8
Controls			
LB1		-	+
BM		-	+
PBL		-	+
CD19$^+$		-	+

No., number of patients analysed; M, methylated; U, unmethylated; +, positive; -, negative.

corresponding gene expression values in children affected by B-ALL (Table III). The results of MSP reactions on the 5' region of *DLX3* show the presence of methylated alleles in 50.84% (30/59) of the samples. In particular *DLX3* is methylated in 70.58% (24/34) of *MLL-AF4* patients and in 38.46% (5/13) of the non-translocated samples. Interestingly, *DLX3* is almost unmethylated (11/12) in specimens with *TEL-AML1* but methylated in leukemia derived cell lines (Fig. 2 and Table III). Real-time quantitative PCR experiments underline that *DLX3* methylation status significantly correlates to down-regulation of the gene in B-ALL paediatric patients (P<0.01) (Fig. 4A). Furthermore, a high methylation incidence of *DLX3* in *MLL-AF4* positive cases is associated with significant down-regulation of expression as compared to specimens with *TEL-AML1* (P=0.0007) (Fig. 4B). Instead, *DLX4* [clustered with *DLX3* in the 17q21 locus (24,25)] which is unmethylated in healthy controls and methylated in 59.32% (35/59) of leukemic samples randomly divided over the three specific genetic groups of patients, does not demonstrate a significant methylation-dependent gene expression regulation (data not shown). The *DLX2* 5' region results unmethylated in both normal and leukemic samples (Fig. 2) thus denying the hypothesis of *DLX2* as epigenetic marker in paediatric B-ALL and underlining the observation that methylation events are locus specific restricted here to the *DLX3-4* locus.

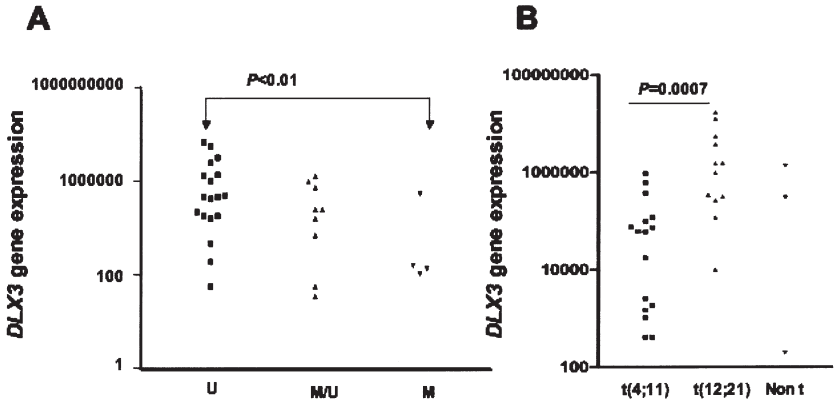

Figure 4. *DLX3* gene expression vs *DLX3* methylation status (A) and *DLX3* gene expression among specific genetic groups of B-ALL patients (B). (A) The x-axis represents the methylation status of *DLX3* alleles in our cohort of patients: U, totally unmethylated; M/U, partially methylated; M, totally methylated. The y-axis represents *DLX3* gene expression value as calculated from RQ-PCR experiments. Each dot corresponds to a B-ALL patient: for each patient *DLX3* gene expression is reported against *DLX3* methylation status. (B) The x-axis reperesents each genetic group in which our paediatric B-ALL patients are split: t(4;11), B-ALL with *MLL-AF4* rearrangement; t(12;21), B-ALL with *TEL-AML1* rearrangement; Non t, B-ALL without rearrangements. The y-axis represents *DLX3* gene expression value as calculated from RQ-PCR experiments.

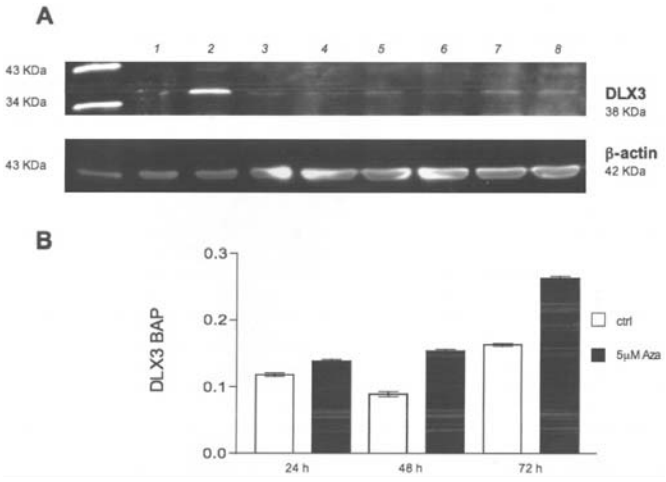

Figure 5. Western blot for DLX3 protein on RS4;11 cell lines during demethylating treatment (A) and corresponding DLX3 protein expression values. (A) DLX3 (upper gel) and ß-actin (lower gel) protein Western blots. Thirty-eight and 42 kDa correspond to DLX3 and ß-actin bands, respectively. Lane 1, normal PB; lane 2, K562 erytropoietic cell line; lane 3, control RS4;11 cells harvested after 24 h; lane 4, RS4;11 cells harvested after 24 h of treatment with 5 μM 5-Aza-2'-dC; lane 5, RS4;11 cells harvested after 48 h; lane 6, RS4;11 cells harvested after 48 h of treatment with 5 μM 5-Aza-2'-dC; lane 7, RS4;11 cells harvested after 72 h; lane 8, RS4;11 cells harvested after 72 h of treatment with 5 μM 5-Aza-2'-dC. (B) y-axis represents DLX3 protein expression levels expressed as BAP. White columns represent DLX3 protein expression values in control experiment with RS4;11 cells harvested after 24, 48 and 72 h. Black columns represent DLX3 protein expression values in experiment with RS4;11 cells harvested after 24, 48 and 72 h of treatment with 5 μM 5-Aza-2'-dC.

Western blot analysis for DLX3 protein on RS4;11 leukemic cell lines during demethylation treatment. Western blot experiments in the RS4;11 laeukemic cell lines (Fig. 5A), where *DLX3* gene expression highly increased during demethylating treatment, confirm that *DLX3* gene expression down-regulation results also in protein down-regulation. In fact, the analysis shows that DLX3 protein expression differs between control experiments with RS4;11 cells and experiments with treated RS4;11 cells (Fig. 5B) increasing in the latter in parallel with decreasing methylation.

Discussion

In a survey for aberrant methylation of *DLX2*, *DLX3* and *DLX4* genes in AL we analyzed cell lines presenting specific chromosomal translocations, fresh normal bone marrow and a normal cell line. First we found that *DLX3* and *4* were methylated in AL cell lines and unmethylated in normal controls, contrary to *DLX2* gene that resulted unmethylated in all cases. This observation prompted us to investigate if this epigenetic feature was related to functional inactivation. *DLX3* gene expression greatly increased upon exposing leukemic cells to the demethylating agent 5-Aza-2'-deoxycytidine. This result proved that the methylation of *DLX3* CpG islands at the 5' moiety, has a functional role and interferes with its expression in B-ALL cells. Also DLX3 protein expression increased with increasing demethylation confirming the functional role of *DLX3* methylation on subsequent events. Since hypermethylation of CpG islands is a common phenomenon in several tumor types and in adult and paediatric ALL where it is associated with poor prognosis and specific patient subgroups (7), we decided to validate our *in vitro* gene expression data, studying two different groups of paediatric B-ALL. We investigated both *DLX3* methylation and gene expression profiles in a genetic cohort of B-ALL carrying *MLL-AF4* translocation: these patients are well known for both their poor prognosis and response to chemotherapy (16). Further, we chose a group of paediatric B-ALL with the *TEL-AML1* chromosomal aberration that represents a cohort of good prognosis patients (17-19), and a control group of B-ALL without translocations. In B-ALL paediatric patients, *DLX3* methylation status significantly correlates to its transcriptional inactivation; furthermore, according to its higher frequency of methylation, *DLX3* expression is lower in *MLL-AF4* cases as compared to those with *TEL-AML1*, suggesting that *DLX3* aberrant methylation could be characteristic for this subgroup of patients and have a specific role in high-risk paediatric B-ALL. *DLX3* unmethylated in specimens with *TEL-AML1* rearrangement, is methylated in *TEL-AML1* leukemia derived cell line probably reflecting the global derangement of the methylation machinery often observed in stabilized cell lines. Moreover these data suggest that methylation could be the reason for immortalization typical of cell lines. Importantly, in spite of the low *DLX4* gene expression level in the methylated *TEL-AML1* samples, we could not observe a statistically significant correspondence between the methylation status of *DLX4* and its down-regulation, likely because of the small number of unmethylated samples present in our population. Even if methylation occurs in both *DLX3* and *DLX4* genes, which are situated in the same locus, and even if they are clustered genes with the same duplication origins (26), they are not co-regulated. This fact probably reflects specific regulatory events for both genes, and locus independent gene-specificity of methylation. Moreover, the opposite methylation pattern of *DLX3* and *DLX4* genes among the two genetic leukaemia subgroups studied, is a further confirmation of the heterogeneity of hypermethylation in leukemia and of the power of hypermethylation to discriminate different genetic and clinical subgroups of patients (7).

Overall survival analysis of the paediatric B-ALL cohort studied here and in particular of *MLL-AF4* patients showed a trend of reduced survival chances in patients characterized by the methylation of *DLX3* 5' region as compared to those with unmethylated ones. However, there is no statistic significance probably because of the small number of patients (data not shown). These data suggests that the methylation of *DLX3* could be responsible for the shorter overall survival of this class of paediatric B-ALL.

The downward trend of survival for *DLX3*-methylated cases and the high percentage of *DLX3* methylation in *MLL-AF4* paediatric patients, who respond poorly to therapy, may well be related to the previously suggested (14) contribution of *DLX3* down-regulation to resistance to apoptosis. The role of *DLX3* down-regulation could be similar to that previously reported for CD10 for high-risk pediatric B-ALL (27). The epigenetic importance of *DLX3* is sustained by the observation that MLL-AF4 paediatric patients have a high incidence of silenced genes frequently methylated in

haematological malignances, that when re-expressed contribute to cell toxicity (28). As previously reported in other studies (10), it is difficult to establish if the down-regulation of *DLX3* methylation is a key event in immature B-cell leukemiogenesis or whether *DLX3* hypermethylation is a consequence of MLL rearrangement. The MT domain common to all MLL fusion proteins, binds specifically unmethylated CpG sequences (29): the first result could be the constant activation of target genes and *DLX3* hypermethylation could be part of a machinery that acts to balance this altered situation. Again, the binding of MLL domain could inhibit the activity of DNA-binding proteins such as transcription factors, thus resulting in a suppressor effect. In this way, *DLX3* methylation could be a downstream effect of an MLL-AF4 target gene. Similar to PML-RAR fusion protein in positive acute promyelocytic leukemia (11), the binding of MLL-AF4 fusion proteins to specific DNA region could be responsible of the recruiting of DNA methyltransferases to hypermethylate the promoter region of downstream targets among which *DLX3*.

In conclusion, our results show for the first time the aberrant methylation of *DLX3* gene and its functional inactivation in the specific cohort of paediatric B-ALL with the MLL-AF4 chromosomal aberration. This finding proposes DLX3 as a new epigenetic candidate marker involved in leukemiogenesis of this high-risk acute lymphoblastic leukemia.

Acknowledgements

Supported by Fondazione Città della Speranza, CNR, and MURST ex. 40 and 60%, Ministero della Salute, AIRC, Fondazione Italiana per la Lotta al Neuroblastoma and FIRB grants: RBNE01TZZ8 and RBAU01935A.

References

1. Pui CH and Evans WE: Acute lymphoblastic leukemia. N Engl J Med 339: 605-615, 1998.
2. Faderl S, Kantarjian HM, Talpaz M and Estrov Z: Clinical significance of cytogenetic abnormalities in adult acute lymphoblastic leukemia. Blood 91: 3995-4019, 1998.
3. Taub JW and Ge Y: The prenatal origin of childhood acute lymphoblastic leukemia. Leuk Lymphoma 45: 19-25, 2004.
4. Bird A: DNA methylation patterns and epigenetic memory. Genes Dev 16: 6-21, 2002.
5. Jones PA and Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev 3: 415-428, 2002.
6. Herman JG and Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349: 2042-2054, 2003.
7. Costello JF, Fruhwald MC, Smiraglia DJ, *et al*: Aberrant CpG-island methylation has non-random and tumor-type-specific patterns. Nat Genet 2: 132-138, 2000.
8. Roman-Gomez J, Jimenez-Velasco A, Castillejo JA, *et al*: Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. Blood 104: 2492-2498, 2004.
9. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
10. Stam RW, den Boer ML, Passier MM, Janka-Schaub GE, Sallan SE, Armstrong SA and Pieters R: Silencing of the tumor suppressor gene FHIT is highly characteristic for MLL gene rearranged infant acute lymphoblastic leukemia. Leukemia 20: 264-271, 2006.
11. Di Croce L, Raker VA, Corsaro M, *et al*: Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 295: 1079-1082, 2002.
12. Panganiban G and Rubenstein JL: Developmental functions of the Distal-less/Dlx homeobox genes. Development 129: 4371-4386, 2002.
13. Ferrari N, Palmisano GL, Paleari L, *et al*: DLX genes as targets of ALL-1: DLX 2,3,4 down-regulation in t(4;11) acute lymphoblastic leukemias. J Leukoc Biol 2: 302-305, 2003.
14. Ferrari N, Paleari L, Palmisano GL, Tammaro P, Levi G, Albini A and Brigati C: Induction of apoptosis by fenretinide in tumor cell lines correlates with DLX2, DLX3 and DLX4 gene expression. Oncol Rep 10: 973-977, 2003.
15. Rush LJ, Raval A, Funchain P, *et al*: Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. Cancer Res 64: 2424-2433, 2004.
16. Di Martino JF and Cleary ML: MLL rearrangements in hematological malignances: lessons from clinical and biological studies. Br J Haematol 106: 614-626, 1999.
17. Shurtleff SA, Buijs A, Behm FG, *et al*: TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in paediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia 9: 1985-1989, 1995.
18. Romana SP, Poirel H, Leconiat M, *et al*: High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia. Blood 86: 4263-4269, 1995.
19. Mclean TW, Ringold S, Neuberg D, *et al*: TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood 88: 4252-4258, 1996.
20. Van Dongen JJ, Macintyre EA, Gabert JA, *et al*: Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 13: 1901-1928, 1999.
21. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
22. Chase MB, Fu S, Hega SB, *et al*: BP1, a homeodomain-containing isoform of DLX4, represses the beta-globin gene. Mol Cell Biol 22: 2505-2514, 2002.
23. Pellegrino MA, Brocca L, Dioguardi FS, Bottinelli R, D'Antona G: Effects of voluntary wheel running and amino acid supplementation on skeletal muscle of mice. Eur J Appl Physiol 93: 655-664, 2005.
24. Shimamoto T, Nakamura S, Bollekens J, Ruddle FH and Takeshita K: Inhibition of DLX-7 homeobox gene causes decreased expression of GATA-1 and c-myc genes and apoptosis. Proc Natl Acad Sci USA 94: 3245-3249, 1997.
25. McGuinness T, Porteus MH, Smiga S, *et al*: Sequence, organization and transcription of the Dlx-1 and Dlx-2 locus. Genomics 35: 473-485, 1996.
26. Sumiyama K, Irvine SQ and Ruddle FH: The role of gene duplication in the evolution and function of the vertebrate Dlx/distal-less bigene clusters. J Struct Funct Genomics 3: 151-159, 2003.
27. Cutrona G, Tasso P, Dono M, *et al*: CD10 is a marker for cycling cells with propensity to apoptosis in childhood ALL. Br J Cancer 86: 1776-1785, 2002.
28. Gutierrez MI, Siraj AK, Bhargava M, *et al*: Concurrent methylation of multiple genes in childhood ALL: correlation with phenotype and molecular subgroup. Leukemia 17: 1845-1850, 2003.
29. Birke M, Schreiner S, Garcia-Cuellar MP, Mahr K, Titgemeyer F and Slany RK: The MT domain of the proto-oncoprotein MLL binds to CpG-containing DNA and discriminates against methylation. Nucleic Acids Res 30: 958-965, 2002.