

# Different molecular mechanisms causing 9p21 deletions in acute lymphoblastic leukemia of childhood

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**Abstract** Deletion of chromosome 9p21 is a crucial event for the development of several cancers including acute lymphoblastic leukemia (ALL). Double strand breaks (DSBs) triggering 9p21 deletions in ALL have been reported to occur at a few defined sites by illegitimate action of the V(D)J recombination activating protein complex. We have cloned 23 breakpoint junctions for a total of 46 breakpoints in 17 childhood ALL (9 B- and 8 T-lineages) showing different size deletions at one or both homologous chromosomes 9 to investigate which particular

sequences make the region susceptible to interstitial deletion. We found that half of 9p21 deletion breakpoints were mediated by ectopic V(D)J recombination mechanisms whereas the remaining half were associated to repeated sequences, including some with potential for non-B DNA structure formation. Other mechanisms, such as microhomology-mediated repair, that are common in other cancers, play only a very minor role in ALL. Nucleotide insertions at breakpoint junctions and microinversions flanking the breakpoints have been detected at 20/23 and 2/23 breakpoint junctions, respectively, both in the presence of recombination signal sequence (RSS)-like sequences and of other unspecific sequences. The majority of breakpoints were unique except for two cases, both T-ALL, showing identical deletions. Four of the 46 breakpoints coincide with those reported in other cases, thus confirming the presence of recurrent deletion hot-spots. Among the six cases with heterozygous 9p deletions, we found that the remaining *CDKN2A* and *CDKN2B* alleles were hypermethylated at CpG islands.

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## Introduction

Unraveling the cause of genomic structural rearrangements has become a hot topic in human genetics. The comprehension of the nucleotide sequences leading to genomic instability might ultimately lead to understand whether deleterious imbalances, both constitutional and acquired, occur at random in people sharing apparently identical genome sequences at a certain locus or are linked to specific haplotypes. In the last years three major mechanisms had been proposed, i.e., non-allelic homologous recombination (NAHR), responsible for recurrent rearrangements, non-homologous end joining (NHEJ), and Fork Stalling

and Template Switching (FoStES), the latter two associated with non-recurrent rearrangements (Gu et al. 2008). Most of these mechanisms had been extrapolated by studying patients with constitutional imbalances. Very few studies have focused on the molecular mechanisms leading to acquired rearrangements although their importance in tumor's initiation and progression is obvious as recently demonstrated by the finding that the acquired JAK2V617F mutation, in myeloproliferative neoplasms, is associated with a germline haplotype block that includes the 3' portion of *JAK2* (Jones et al. 2009; Olcaydu et al. 2009; Kilpivaara et al. 2009). This finding indeed implies the existence of a strong, but uncharacterized, interaction between somatic and germline genetics (Campbell 2009). We cloned 23 breakpoint junctions, for a total of 46 breakpoints, in 17 ALL of childhood (9 B- and 8 T-lineages; 2–4 breakpoints per case according to the presence of different heterozygous/homozygous deletions) showing non-recurrent deletions at one or both homologous chromosomes 9; all deletions contained *CDKN2A*, most also involved *CDKN2B*. These 9p21.3 genes encode three cell cycle inhibitory proteins: p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, and p14<sup>ARF</sup> (Gil and Peters 2006). Homozygous deletions removing p16 or heterozygous deletions with inactivation of the normal allele occur in a variety of cancers (Drexler 1998; Ruas and Peters 1998; Orlow et al. 1999; Bertin et al. 2003; Irving et al. 2005; Murao et al. 2006; Kohno and Yokota 2006; Strefford et al. 2007; Usvasalo et al. 2008; Bungaro et al. 2009). Therefore, this locus is considered a paradigm of the two-hit hypothesis (Knudson 1971). In the last years, both lymphoid leukemias and other types of tumors have been analyzed for the 9p21 deletion (Kitagawa et al. 2002; Florl and Schulz 2003; Sasaki et al. 2003; Kohno and Yokota 2006). Kohno and Yokota (2006) mapped the breakpoints of 21 lymphoid leukemia cases, 21 lung cancer cases, and 32 other solid cancer cases at a 10-kb resolution. They found that regions of breakpoint clustering were different in different malignancies and that the majority of the 20 cloned breakpoints in lymphoid leukemia clustered at a few breakpoint sites, each <15-bp in size. The sites were flanked by sequences similar to the heptamer-RSSs which are targeted by the RAG complex in V(D)J recombination, as previously shown also by Cayuela et al. (1997).

While screening by genome-wide array-CGH a bone marrow sample of 65 childhood B- and T-ALLs, we detected 18 cases (27.7%) having either heterozygous or homozygous 9p21 deletions. We then performed a detailed analysis of breakpoint junctions in only 17 of them (9 B- and 8 T-ALLs) in order to better understand which particular sequences make the 9p21 region susceptible to interstitial deletions. Moreover, we have studied, by CpG island methylation analysis, the activation/inactivation state of *CDKN2A* and *CDKN2B* in heterozygous deletions.

## Materials and methods

### Patients

Sixty-five pediatric patients, aged between 4 months and 18 years (median age 5 years and 3 months), were included in the first screening through whole-genome array-CGH at a 100-kb resolution (Kit 44B, Agilent Technologies, Santa Clara, CA). Of the 65 children, 53 were affected by B-ALL, whereas 12 had T-lineage ALL (see Supplementary Table 1 for patients characteristics). All patients were diagnosed and treated in a single center (Oncoematologia Pediatrica Fondazione IRCCS Pavia, Italy) according to the front-line protocol of the Italian Association of Pediatric Hematology and Oncology (AIEOP) for childhood ALL (AIEOP LLA 2000) or the relapse protocol (AIEOP LLA REC 2003), with the only exception of a 4-month-old child who received chemotherapy according to the Interfant 99 protocol (Pieters et al. 2007). The study received approval by the local Institutional Review Board, and informed consent was obtained from all patients' parents. Patients were censored as of 1 August 2007, the median follow-up time being 3.3 years (range 3–78 months).

The diagnosis of ALL was based on morphological, cytochemical, and immunophenotypical criteria. Eligible patients had fewer than 3% blasts positive for myeloperoxidase or Sudan black, and were negative for non-specific esterase according to the French–American–British criteria. Positivity criteria for immunophenotyping were defined according to the BFM-family criteria, with the limits of 20% for surface antigens and 10% for intracellular markers (van der Does-van den Berg et al. 1992). The presence of the translocations *t*(9;22) and *t*(4;11) was systematically screened by reverse transcriptase-polymerase chain reaction (RT-PCR) in a centralized laboratory (van Dongen et al. 1999). CR was defined as the absence of physical signs of leukemia or detectable leukemia cells on blood smears, a BM with active haematopoiesis and fewer than 5% leukemia blast cells and normal cerebrospinal fluid.

Patients with 9p21 deletions were selected from the first screening: ten were affected by B-ALL and 8 with T-ALL.

### Customized array-CGH

Each childhood ALL sample was analyzed with a 44B Agilent platform for genomic array having in average an oligomer every 35-kb. Because the minimum positive call was considered three consecutive oligomers with a log<sub>2</sub> ratio different from zero [cut-off log<sub>2</sub> ratios: –1 and –4 for constitutional heterozygous and homozygous deletions, respectively; +0.5 for constitutional duplications; +1 for constitutional triplication; a log<sub>2</sub> ratio of –0.3 is representative of a cellular mosaic of about 50%, as

demonstrated by dilution experiments (data not shown)], the true resolution of the platform was about 100-kb. As reference DNA we used a pool of female or male lymphocytes (Promega, Milan, Italy). All CNVs not reported in the database of genomic variants (<http://projects.tcag.ca/variation/>) and not already known to be associated with leukemia were confirmed as de novo or acquired with a new array experiment using patient's saliva as reference DNA. Each case with 9p21 deletion was reanalyzed on a customized platform generated using the eArray software (<http://earray.chem.agilent.com/>); the probes (60-mer oligonucleotides) were selected from those available in the Agilent database and are specific for the 9p21 region. The array was made of 44,000 probes (custom 44K, Agilent Technologies) of which 19,000 represented the region under study (from 19 to 25 Mb), allowing molecular profiling of 9p21 aberrations with a resolution of ~1 and ~10-kb in single copy and repeated regions, respectively. DNA of every ALL sample was extracted from bone marrow (BM) mononuclear cells with at least 80% of blasts (range 80–100%, median value 90%, evaluated morphologically and through flow cytometry), after gradient separation, or peripheral blood (PB, only when the presence of leukemia blasts in the periphery was equal to or superior than 80%) using a QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's protocol. Test DNA (from leukemia BM or PB leukemia cells, 500 ng) and control DNA (Human Genomic DNA Female or Male, Promega, Madison, WI, 500 ng) were double-digested with *RsaI* and *AluI* (Promega) for 1 h at 37°C. After digestion, samples were incubated at 65°C for 20 min to inactivate the enzymes then labeled by random priming (Agilent Technologies) for 2 h using cyanine5-dUTP for the test DNA and cyanine3-dUTP (Agilent Technologies) for the control DNA. Labeled DNAs were incubated at 65°C for 10 min and then cleaned with Microcon YM-30 filter units (Millipore, Billerica, MA). Purified samples were brought to a total volume of 21 µl in 1×TE (pH 8.0, Promega), and yield and specific activity were determined for each sample using a NanoDrop ND-1000 UV–VIS Spectrophotometer (Agilent Technologies). The appropriate cyanine 5- and cyanine 3-labeled samples were combined in a total volume of 39 µl. After sample denaturation and pre-annealing with 5 µl of Human Cot-1 DNA (Invitrogen, Carlsbad, CA), hybridization was performed at 65°C with shaking for 24 h. After two washing steps, the array was analyzed on the Agilent scanner (DNA Microarray Scanner with Sure Scan High-Resolution Technology, Model G2565CA, Agilent Technologies) and then by the Feature Extraction software (v9.1). Graphical overview was obtained using the CGH analytics software (v3.4.27). The positions of oligomers refer to the Human Genome March 2006 assembly (hg18).

Results were also visualized using the UCSC Genome Browser website (<http://genome.ucsc.edu/>).

#### Breakpoint mapping

Breakpoints were cloned by long range PCR using primers that recognize sequences flanking the distal and proximal breakpoints, respectively, based on the customized array-CGH data. All primer sequences are available on request.

Long-range PCRs were performed with JumpStart Red ACCUTaq LA DNA polymerase (Sigma-Aldrich, Steinheim, Germany) and the following protocol: 30 s at 96°C, 35 cycles of 15 s at 94°C/20 s at 58°C/10 min at 68°C, 5 min final elongation time. Sequencing reactions were performed with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and run on an ABI Prism 3130xl Genetic Analyzer.

#### DNA methylation assay

Sodium bisulfite conversion of unmethylated cytosine residues to uracil in DNA samples (700 ng) was performed using a DNA Methylamp™-One step-DNA modification kit (Epigentek, Brooklyn, NY) according to the manufacturer's protocol. Primers for *CDKN2A* and *CDKN2B* were designed with CpG Ware software ([http://apps.serologicals.com/cpgware/dna\\_form2.html](http://apps.serologicals.com/cpgware/dna_form2.html)) to specifically amplify each of the sequences, based upon chemically induced differences. Methylation-specific polymerase chain reaction (MS-PCR) amplification was carried out using primers specific for either wild-type, methylated or unmethylated DNA (see Supplementary Table 4 for details). The PCR mixture contained 10× *Taq* reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 50 mM MgCl<sub>2</sub> (final concentration 1.5 mM), 10 mM dNTPs mixture (final concentration 0.2 mM each), 20 mM primers (300 ng each per reaction), 1.5 units of *Taq* DNA polymerase (all material from Invitrogen, Carlsbad, CA) and bisulfite-modified (~100 ng) or unmodified DNA (~100 ng) for a final volume of 30 µl. PCR specific for unmodified DNA also included 0.5 M betain (Sigma-Aldrich, Steinheim, Germany). Amplification was carried out in a thermocycler, with the following PCR cycling profile: preheating at 96°C for 5 min, followed by 95°C for 30 s, annealing temperature for 30 s (see Supplementary Table 4 for each annealing temperature), 72°C for 30 s for 35 cycles, and a final extension at 72°C for 7 min. The PCR samples were loaded onto a 2.5% agarose gel, stained with ethidium bromide, and directly visualized under ultraviolet (UV) illumination. MS-PCR was also performed on five positive controls with 9p21 deletions, detected by whole-genome array-CGH, from other different type of cancers and on the same number of negative controls.

To confirm the methylation state of *CDKN2A* and *CDKN2B*, an aliquot of each PCR product was cut with *Bst*UI (New England Biolabs, Beverly, MA), which recognizes the sites of methylation (5'...CGCG...3' and 3'...GCGC...5'), for 1 h at 60°C, then loaded on a 2.5% agarose 1×TAE gel. Cleavage occurs if the CGCG sequence has been retained during bisulfite conversion due to the methylated status of the cytosine residues. A second aliquot of the PCR products was purified with UltraClean PCR Clean-up (MoBio. Labs, Carlsbad, CA) according to the manufacturer's protocol and sequencing reactions were performed by automated DNA Sequencing (PE Applied Biosystem).

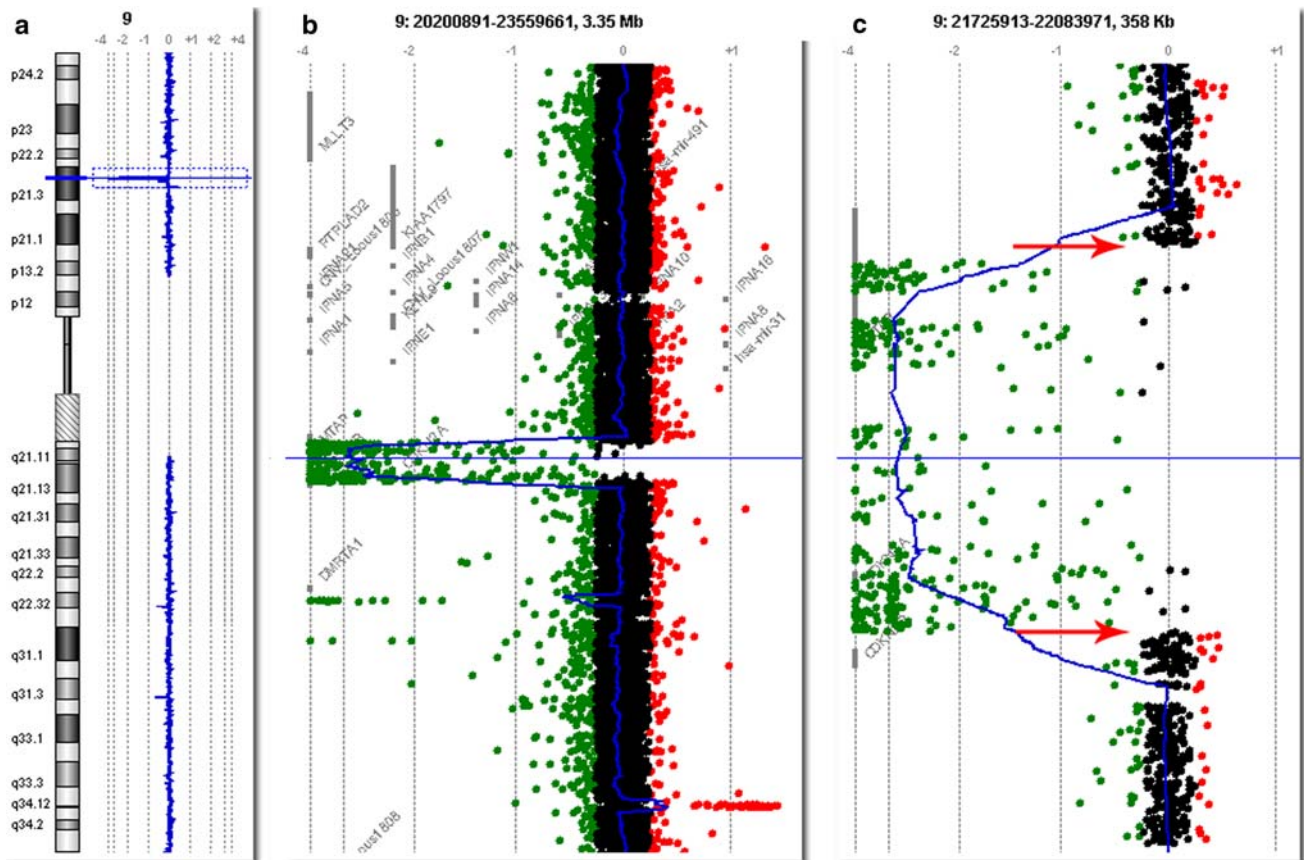
## Results

Genome-wide array-CGH analysis detected several copy number aberrations in 90.7% (59/65) of the cases (Supplementary Table 2). The most frequent imbalances involved 6q16.2-q21 (5 cases), 12p13.1-p13.2 (13 cases, of which 9 encompassing *CDKN1B*), and 14q32.33 encompassing the IgH locus (30 cases). All these imbalances are beyond the aims of this study but they are summarized in Supplementary Table 2. The frequency of homozygous/heterozygous 9p21 deletions was 27.7% (18/65) and was higher in T-ALLs than in B cell precursor ALLs [66.7% (8/12) vs. 18.9% (10/53)]. In most samples, the log<sub>2</sub> ratio was between -0.3 and -0.8 for heterozygous and between -2 and -3.5 for homozygous deletions, due to normal cells contamination. Customized array-CGH experiments confirmed the data obtained on the 44K platform and allowed us to better characterize the size of each deletion (Fig. 1; Supplementary Table 3). The region between the last normal and the first deleted oligomers ranged between 51 bp and 7.8 Kb. Deletion size and location were different in all cases, except for two cases characterized by a deletion with proximal and distal identical breakpoints (888-06 and 314-07, Supplementary Table 3). Cloning of the breakpoint junctions allowed us to characterize the precise size of each deletion (Fig. 2a) and to identify the sequences flanking each breakpoint junction (Table 1). All deletions always spanned at least *CDKN2A* and either *CDKN2B* or *MTAP* (Fig. 2b). In one case (1329-07), the shorter *CDKN2A* isoforms were not deleted. When heterozygous deletions of *CDKN2A*, or both *CDKN2A* and *CDKN2B*, were present (6 cases: 888-06, 46-07, 1623-06, 1329-07, 886-07 and 547-07; Fig. 2a; Supplementary Table 3), the remaining allele was always methylated at its promoter CpG island (Fig. 3). Twenty-three 9p21 breakpoint junctions (for a total of 46 breakpoints) were cloned from only 17 childhood acute lymphoblastic leukemia cases, 8 T- and 9 B-ALLs (Table 1), because the cloning

failed for one B-ALL (case 884-07). Junctions were cloned from 11 cases with two breakpoints (either heterozygous deletions or identical homozygous deletions), 4 cases with four breakpoints (heterozygous/homozygous deletions with different breakpoints) and 2 cases (888-06 and 314-07) with multiple deletions on the same allele for a total of four breakpoints per case. One B-ALL case (402-06), analyzed both at onset and relapse, presented identical 9p21 deletion breakpoints. Only one T-ALL case (1460-07, BP1) showed a breakpoint junction with an overlap of six nucleotides, evidence of microhomology-related rearrangement. Two junctions, one from a T-ALL case (1562-06) and one from a B-ALL case (1327-07) had neither insertions nor nucleotide overlaps. A de novo insertion 1–12 nucleotides in size (average 4.8 nucleotides, T cells 4.4 nucleotides, B cells 5.3 nucleotides) was present at all other breakpoint junctions. One T cell leukemia (219-07, BP1) had at its proximal breakpoint a 227 bp inversion preceded by three extra nucleotides not present in the consensus sequence. The second allele from the same subject (219-07, BP2) contained a 427 bp insertion from chromosome 4q32.2. A B-ALL case (1623-06) carried at its distal breakpoint a 253 bp inversion bracketed by 15 bp of duplicated inverted sequence and followed by seven extra nucleotides not present in the genomic consensus sequence. Two T-ALL cases (547-07 and 217-07) had essentially the same junctions, with distal breakpoints one base apart in an Alu repeat and proximal breakpoints in the same (TG)<sub>n</sub> repeat; both cases had de novo insertions: AGG in one, TTTAAGTAAG in the other. A third B-ALL case (46-07) had its proximal breakpoint in the same (TG)<sub>n</sub> repeat. Two cases (888-06 and 314-07) carried two deletions on the same chromosome, one spanning *CDKN2A*, the other more proximal (BP2: 22486528 to 22494344). Sequence analysis of the second deletion showed that both cases had the same breakpoints. Deletion-specific PCR amplification of 21 healthy controls (not shown) demonstrated that the deletion was present in 12 of them and should, therefore, be considered a genomic polymorphism. Copy number variations in the same interval are listed in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) (Iafate et al. 2004) as Variation\_37473 (Cooper et al. 2008) and Variation\_7739 (de Smith et al. 2007).

All repeated sequences located within 60 bp from the breakpoints are listed in Table 1. Twenty-seven of the 46 breakpoints (58.7%) are actually inside a repeat. The more frequent family of repeats was LINE (12 breakpoints; T: 3; B: 9); SINE (5 breakpoints; T: 3; B: 2), simple repeats (4 breakpoints; T: 3; B: 1), DNA (3 breakpoints; T: 2; B: 1) and LTR (3 breakpoints; T: 2; B: 1) were also represented. In 11 junctions, repeats were present at both breakpoints; in nine junctions, at





**Fig. 1** An example of custom array-CGH results: case 217-07. **a** Chromosome 9 profile with 9p21 deletion indicated by the *box*, **b** enlargement of the 9p21 region showing a homozygous deletion, **c** further enlargement

of the locus under study; the *arrows* point to the edge between normal and deleted oligomers

one breakpoint; in three junctions, no repeats were involved.

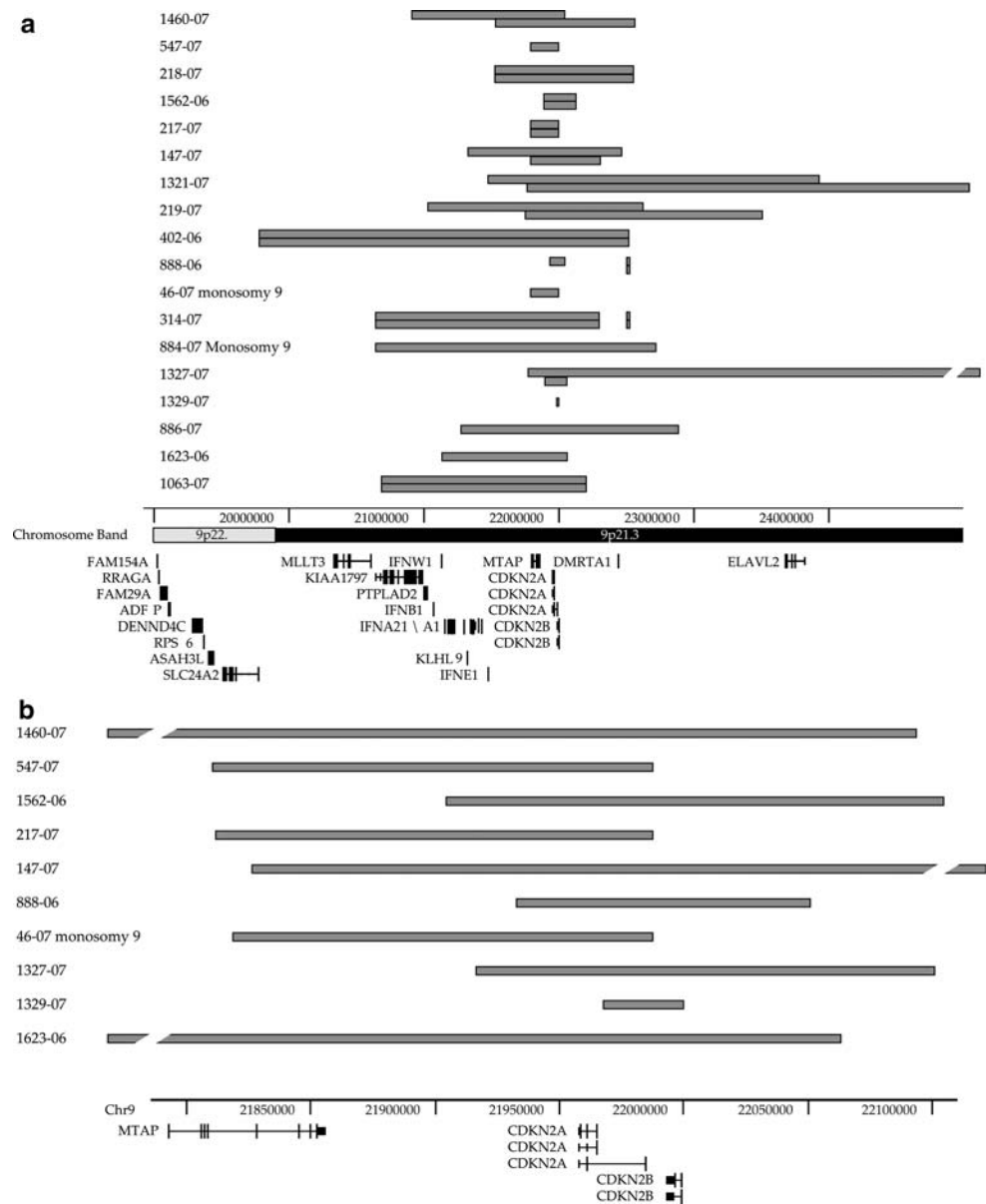
To assess the involvement of illegitimate V(D)J recombination in 9p21 deletions, RSS-like sequences were examined. The 23 bp sequence flanking each breakpoint was visually searched for 5'-CAC trinucleotides in the forward direction and the 3'-GTG trinucleotides in the reverse direction. This choice was due to the fact that the CAC trinucleotide in the heptamer-RSS (CACAGTG) is the only part of the RSSs indispensable for V(D)J recombination (Hesse et al. 1989; Akamatsu et al. 1994) and nucleotide loss as a result of V(D)J recombination does not extend over 20 bp (Nobori et al. 1996). Sequences similar to the nonamer RSS (ACAAAACC) were also considered. RSSs sequences were found at 12/24 breakpoints in our T-ALL cases and in 12/22 breakpoints in B-ALLs (Table 1).

## Discussion

In the past few years, several studies have focused on understanding the origin of deletions and duplications leading to both benign and pathogenic copy number variations

(CNVs). Some constitutional CNVs are obviously of meiotic origin (Turner et al. 2008; Gu et al. 2008). For what concerns acquired CNVs, it seems now very clear that they are not limited to tumor processes. The concept of a stable somatic genome has been challenged by the finding of extensive genomic copy number variation in mouse embryonic stem cells suggesting a similar situation in humans (Liang et al. 2008). These data reinforced previous studies showing that mitotic ectopic exchanges occurring in the soma and germ line mainly contribute to de novo occurrence of human alpha-globin genes duplications, whereas meiotic exchanges between homologous chromosomes generate a minority of sperm duplications (Lam and Jeffreys 2007). Somatic CNVs occurrence has also been demonstrated by the finding that identical twins can differ in CNV (Bruder et al. 2008). Deletions at 9p21 deserve attention because removal of the p16 tumor suppressor gene is a genetic alteration frequently observed in a variety of human cancers (Drexler 1998; Ruas and Peters 1998; Orlow et al. 1999; Bertin et al. 2003; Irving et al. 2005; Murao et al. 2006; Kohno and Yokota 2006; Strefford et al. 2007; Usvasalo et al. 2008; Bungaro et al. 2009). Kohno and Yokota (2006) reported that DNA double strand breaks (DSBs) triggering

**Fig. 2 a** Graphic representation of the 9p21.3 deletions in all 18 cases. *Grey lines* represent the deleted region of every allele of each case. Overlap of two lines for the same case indicates a homozygous deletion. **b** Detailed map of the *MTAP/CDKN2A/CDKN2B* region. The ten cases with homozygous/heterozygous deletions, whose breakpoints (at least one) map between 21,800,000 and 22,100,000 bp, are reported



9p21 deletions in lymphoblastic leukemia lines are formed at a few defined sites by the illegitimate action of the recombination-activating genes (RAG) protein complex in V(D)J recombination, as previously reported (Cayuela et al. 1997), while 9p21 DSBs in solid tumor lines are formed at unspecific sites by microhomology-mediated mechanisms.

In our whole-genome array-CGH screening of 65 childhood ALL, we found 9p21 deletions in 18 cases, corresponding to a frequency of 27.7%, comparable to the 29% reported by Bungaro et al. (2009) in a pediatric ALL sample analyzed with a similar approach. In the present study, 23 breakpoint junctions of 9p21 deletions were cloned from 17 acute lymphoblastic leukemias of childhood, consisting of 8 T- and 9 B-lineage samples, for a total of 46 break-

points (at least two breakpoints for each case; some cases had different breakpoints in each homologue). Our aim was to examine which specific sequences, if any, are responsible for the deletion and if genomic architecture acts as susceptibility factor for the deletion itself.

The deletions we analyzed vary between 35 kb and 17 Mb in size: 33% of them are homozygous, and 44% smaller than 1 Mb. Breakpoint junction cloning revealed that half of the breakpoints (11/24 and 12/22 in T- and B-ALL cases, respectively) were flanked by sequences similar to the heptamer-recombination signal sequences (heptamer-RSSs), which are targeted by the RAG complex in V(D)J recombination. This mechanism, identified in 9p21 deletions since 1997 (Cayuela et al. 1997), is applicable to about half of the childhood ALLs, at odds with a recent

**Table 1** Sequences of all characterized breakpoints in childhood ALLs

Case	ALL <sup>a</sup>	Breakpoint <sup>b</sup>	Position <sup>c</sup>	Sequence <sup>d</sup>	Repeats <sup>f</sup>	
1460-07	T	Distal BP1	chr9:20881065–20881130	ttaatcatcatgctctgtgtgatgctctatg       ttaatcatcatgctctgtgtgatgctctatg	at       ATTAAT ATTAAT	Tigger1
		Junction 1				
		Proximal BP1	chr9:22098125–22098190	TCCTCTCTACTTTGTTTCTTATAATAGA		
		Distal BP2	chr9:21552789–21552848	AATAAGCTTTTATGTCGGAAGTTAGCAGATA       AATAAGCTTTTATGTCGGAAGTTAGCAGATA		
		Junction 2			<b>CG<sup>e</sup></b>	
547-07	T	Distal BP	chr9:21815532–21815591	gtcattttgataatggcattgtggcctgg       gtcattttgataatggcattgtggcctgg		LTR16D/L1MD L1MA7/AluJB
		Junction			<b>AGG</b>	
		Proximal BP	chr9:21983793–21983852	TCCTACGtgggtgtgtgtgtgtgtgtgtgtg		
218-07	T	Distal BP	chr9:21527711–21527770	aatactttacagacaagcaaatgctgagag       aatactttacagacaagcaaatgctgagag		LIPA2
		Junction			<b>GG</b>	
		Proximal BP	chr9:22518231–22518287	AGTTCCTCCAAGGCAGTATGCTACTTCCTCTA		
1562-06	T	Distal BP	chr9:21909987–21910046	gaactgtctctaatcctaattgaacagaa       gaactgtctctaatcctaattgaacagaa		MGR75B
		Junction				
		Proximal BP	chr9:22105710–22105769	ATTATCAGCTTGGATCAGATGGACTTGGGG		
217-07	T	Distal BP	chr9:21815531–21815590	tgctcattttgataatggcattgtggcctgg       tgctcattttgataatggcattgtggcctgg		L1MA7/AluJB
		Junction			<b>TTTAAGTAAG</b>	
		Proximal BP	chr9:21983806–21983865	gtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg		
147-07	T	Distal BP1	chr9:21328422–21328481	aacatgacggttttacatttaggaaaagaaa       aacatgacggttttacatttaggaaaagaaa		L2
		Junction 1			<b>TCCTTAGTCCCAA</b>	
		Proximal BP1	chr9:22440703–22440762	ATTTAGGAAGATCCCACACACCAAGATAG		
		Distal BP2	chr9:21831409–21831468	gctaaccagagatcctgaaatgtccacat       gctaaccagagatcctgaaatgtccacat		
		Junction 2			<b>TAAT</b>	
1321-07	T	Distal BP1	chr9:21435322–21435381	GAAGAGCCTGAAATTGCAGAGGAGTCTCTT       GAAGAGCCTGAAATTGCAGAGGAGTCTCTT		LIPB1
		Junction 1			<b>GTGT</b>	
		Proximal BP1	chr9:23928414–23928473	TAAATCTTTTATGACTTTGGCTCAAGTGG		
		Distal BP2	chr9:21788241–21788300	CTCTTTCACTGAGGAGGTATAGGGTTTCAG       CTCTTTCACTGAGGAGGTATAGGGTTTCAG		
		Junction 2			<b>TCAT</b>	
219-07	T	Distal BP1	chr9:21055684–21055743	AACaaatttaatttttaaatattgtaattta       TTATATTCAAAGAGATGCTGGAACGAGGAA		AT-rich
		Junction 1			<b>CCC chr9:22709999–22710226 (rev)</b>	
		Proximal BP1	chr9:22710197–22710256	AGACATCTGATTATAATCAGTACTCTATTT		
		Distal BP2	chr9:21738571–21738630	GTGAAGAACTGAGCTTCCCTAGATCTTatc       GTGAAGAACTGAGCTTCCCTAGATCTTatc		
		Junction 2			<b>chr4:163284405–163284832</b>	
402-06	B	Distal BP	chr9:23484206–23484265	caaacttctattttgtctaaaggaatttaag       tagggacatggatgaagctggaaccatca		LIPA4
		Junction			<b>GGCG</b>	
		Proximal BP	chr9:19785967–19786027	tagggacatggatgaagctggaaccatca       tagggacatggatgaagctggaaccatca		
402-06	B	Distal BP	chr9:22514478–22514537	tatttatactctataattcatttagcgaa       gtgtatcacatatttctcatgataaaaaaga		L3
		Junction				

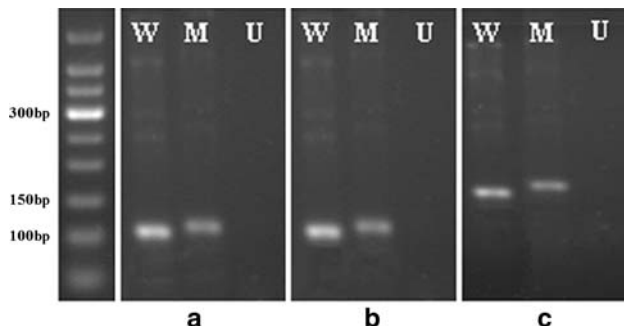
estimate (Kohno and Yokota 2006). In physiological V(D)J recombination, several nucleotides are often added at DNA ends before joining to increase the diversity of immunoglobulin and T cell receptor gene products (Meier and Lewis 1993). It must be noted that we found nucleotide insertions, from 1 to 12 nucleotides, at the breakpoint junctions of 9p21 deletions in the majority (20/23) of the junctions, not just where RSSs-like heptamer sequences were present, in accordance with the finding that 10–20% of non-homologous junction in mammalian cells have nucleotides inserted at the junction (Roth et al. 1989). In one case (219-07), the inserted sequence was longer (427 bp) and

came from 4q32.3, underscoring the complexity of rearrangement events in cancer cells (Bignell et al. 2007).

Although many studies on cancer-related structural variations report microhomology at the junctions in a large proportion of the rearrangements (Bignell et al. 2007), we found only a single case with an overlap of six nucleotides (1460-07). In our cohort, the great majority of the junctions have no homology between distal and proximal ends (22/23, 95%). The breakpoints fall only partially into previously identified cluster sites (Kohno and Yokota 1999) with two notable exceptions. In one case (1329-07), the proximal breakpoint overlaps the breakpoint of three previously analyzed lines,







**Fig. 3** MS-PCR analysis for *CDKN2A* and *CDKN2B* showing the products from wild-type/unmodified DNA (*W*), unmethylated bisulfite-modified DNA (*U*) and methylated bisulfite-modified DNA (*M*). On the left: molecular weight marker (GelPilot 50 bp). **a**, **b**, and **c** MS-PCR products using specific primers for p16INK4a, p14ARF and p15INKb, respectively, from case 1623-06

structure of the heterozygous deletion and the ability of the remaining allele to undergo promoter methylation.

In conclusion, our data show that different molecular mechanisms underlie the 9p21 deletion in childhood ALL. In half of the cases, the deletion is mediated by ectopic V(D)J recombination at RSSs-like sequences. Microhomology-mediated deletion, reported as frequent in cancer structural variations, was found only in one case. In the remaining cases, repeated sequences, including (GT)<sub>n</sub>, with left-handed Z-DNA conformation potential, were detected at the breakpoint junctions. Insertions are common at breakpoint junctions. We identified only two cases sharing identical deletions. Four of our 46 breakpoints coincide with previously reported breakpoints, thus confirming the presence of recurrent deletion hotspots. In general, most of the breakpoints are non-recurrent and generate deletions of variable size. Our data partially answer the unsolved questions stressed by Kohno and Yokota (2006) showing that the chromatin architecture that makes DNA susceptible to DSBs is similar to the one detected in constitutional genomic disorders, with repeated sequences regions of DNA with non-B conformation potential playing a crucial role in predisposing to genome instability (Bacolla and Wells 2004; Wells 2007). However, we cannot exclude that deletion occurrence may also be influenced by other factors, such as interaction between *cis*- (Jones et al. 2009; Olcaydu et al. 2009; Kilpivaara et al. 2009) and *trans*-acting elements, as recently suggested in mouse meiotic recombination (Parvanov et al. 2009). Further studies are necessary to find out which haplotypes, if any, predispose to these somatic rearrangements.

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