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ORIGINAL ARTICLE

PAX5 mutations occur frequently in adult B-cell progenitor acute lymphoblastic leukemia and *PAX5* haploinsufficiency is associated with *BCR-ABL1* and *TCF3-PBX1* fusion genes: a GRAALL study

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Adult and child B-cell progenitor acute lymphoblastic leukemia (BCP-ALL) differ in terms of incidence and prognosis. These disparities are mainly due to the molecular abnormalities associated with these two clinical entities. A genome-wide analysis using oligo SNP arrays recently demonstrated that *PAX5* (paired-box domain 5) is the main target of somatic mutations in childhood BCP-ALL being altered in 38.9% of the cases. We report here the most extensive analysis of alterations of *PAX5* coding sequence in 117 adult BCP-ALL patients in the unique clinical protocol GRAALL-2003/GRAAPH-2003. Our study demonstrates that *PAX5* is mutated in 34% of adult BCP-ALL, mutations being partial or complete deletion, partial or complete amplification, point mutation or fusion gene. *PAX5* alterations are heterogeneous consisting in complete loss in 17%, focal deletions in 10%, point mutations in 7% and translocations in 1% of the cases. *PAX5* complete loss and *PAX5* point mutations differ. *PAX5* complete loss seems to be a secondary event and is significantly associated with *BCR-ABL1* or *TCF3-PBX1* fusion genes and a lower white blood cell count.
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Introduction

PAX5 (paired-box domain 5) is the guardian of the B-cell identity as stated in a recent review.[1] This transcription factor belongs to the family of paired-box domain transcription factors.[2] Its expression is initiated during early stages of B-cell differentiation beginning at the pro-B stage,[3] and is turned off to allow terminal B-cell differentiation.[4] The *Pax5* homozygous deletion in murine models leads to the trans- or de-differentiation of B-cells into several other hematopoietic cell lineages.[5–7] *PAX5* is thus involved both in the maintenance of B-cell identity and in the control of terminal B-cell differentiation.

Deregulations and mutations of key differentiation factors are frequently found in lymphomas and leukemias. Translocations associated with hematologic malignancies involving *PAX5* exemplify PAX5 dual function. On one hand, the t(9;14)(p13;q32) translocation brings the potent enhancer of the *IGH* gene close to the *PAX5* promoter leading to an aberrant expression of a normal PAX5 protein.[8,9] This translocation is recurrent in small plasmacytoid B-cell lymphocytic lymphomas and diffuse large B-cell lymphomas.[10] It emphasizes the importance of *PAX5* downregulation during terminal B-cell differentiation.[9] On the other hand, *PAX5* translocations have also been associated with a block of early B-cell differentiation because the PAX5-ETV6 chimeric protein, product of the dic(9;12)(p13;p13), is associated with B-cell progenitor acute lymphoblastic leukemia (BCP-ALL).[11] Additional PAX5 fusion partner genes have been identified as *HIPK1* (chromosomal band 1p13),[12–14] *LOC392027* (7p12.1),[15] *AUTS2* (7q11.1),[13] *POM121* (7q11),[14] *ELN* (7q11),[16] *JAK2* (9p24),[14] *SLCO1B3* (12p12),[15] *DACH1* (13q21),[14] *PML* (15q24),[17] *ZNF521* (18q11.2),[12] *ASXL1* (20q11.1),[15] *C20orf112* (20q11.1),[13–15] *KIF3B* (20q11.21)[15] and *BRD1* (22q13).[14] PAX5-ELN,[16] PAX5-FOXP1[12] and PAX5-ETV6[12] act as constitutive repressors of the remaining *PAX5* allele product, explaining the block of B-cell differentiation.

To further emphasize the function of PAX5 in B-cell differentiation and oncogenesis, it has recently been reported that the *PAX5* gene is the most frequent target of somatic mutations in childhood BCP-ALL, being altered in 38.9% of the cases.[12] These mutations consist of partial or complete hemizygous deletions, homozygous deletions, partial or complete amplifications, point mutations or fusion genes.[12] Some of these mutants have a dominant negative role on wild-type PAX5.[12]

Adult and childhood BCP-ALL can be considered as two distinct pathological entities in terms of pathogenesis and prognosis.[18] Although 80% of the children with ALL can be

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cured, only 30% of the adults achieve long-term disease-free survival (DFS).[18] Chromosomal abnormalities associated with these pathologies are different in term of occurrence. For example, *BCR-ABL1* fusion gene is associated with a poor long-term response to chemotherapy and is the most common rearrangement associated with adult BCP-ALL accounting for 25% of the cases whereas it is very rarely found in pediatric BCP-ALL. Conversely, *ETV6-RUNX1* fusion gene and high hyperdiploidy are associated with a good prognosis and account for half of the child BCP-ALL but rarely occur in adult BCP-ALL.[18] Apart from these three biological entities, other categories such as *TCF3-PBX1* fusion gene (also called *E2A-PBX1*) or *MLL* rearrangements or normal karyotypes occur with similar frequencies.[18]

Despite these discrepancies between childhood and adult BCP-ALL, we report here the high frequency of *PAX5* mutations in a unique cohort of adult BCP-ALL treated according to the protocols of the GRAALL Intergroup (Group of Research on Adult Acute Lymphoblastic Leukemia), GRAALL-2003 (*BCR-ABL1*-negative BCP-ALL[19]) and GRAAPH-2003 (*BCR-ABL1*-positive BCP-ALL[20]). The deletion of one copy of *PAX5* was found to be significantly associated with *BCR-ABL1* or *TCF3-PBX1* fusion genes and a lower white blood cell (WBC) count.

Material and methods

GRAALL-2003 and GRAAPH-2003 clinical protocols

The GRAALL includes the former LALA (Leucémies aiguës lymphoblastiques de l'adulte), the GOELAL (Groupe Ouest et Est des Leucémies Aigües Lymphoblastiques), and the Swiss Group for Clinical Cancer Research. The GRAALL-2003/GRAAPH-2003 study was a risk-adapted prospective phase 2 trial, conducted in 70 centers in France, Belgium and Switzerland (ClinicalTrials.gov Identifier: NCT00222027). Patients aged 15- to 60-year old with a newly diagnosed ALL were eligible. Between November 2003 and November 2005, 300 patients entered the study. Written informed consent was obtained from all patients or from the parents of those aged less than 18-year old before enrollment. The study was approved in March 2003 by the institutional review board of Purpan hospital, Toulouse, France, and was conducted in accordance with the Declaration of Helsinki Principles. All patients first received a common steroid prephase. Corticoresistance was defined as a peripheral blood blast cell count higher than 1.0×10^9 per liter at the end of this 7-day prephase. Chemoresistance was defined as a percentage of blasts higher than 5% at day 8 of the induction course. Patients were eligible for the GRAAPH-2003 study if they were diagnosed with a *BCR-ABL1*-positive ALL defined as ALL carrying the t(9;22) translocation on standard karyotype and/or fluorescent *in situ* hybridization (FISH) analysis and/or positivity for *BCR-ABL1* fusion transcript detected by PCR analysis. The GRAAPH-2003 study evaluated the efficacy of imatinib mesylate combined to chemotherapy.[20]

PAX5 *exon copy number*

Quantification of *PAX5* copy number was performed by quantitative PCR on genomic DNA from cells of 117 BCP-ALL included in the GRAALL-2003/GRAAPH-2003 trial and for which DNA material was available. Quantitative PCR was performed for each of the 10 exons in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), specific *PAX5* exon primers (Supplementary Table S1) and normal tonsil DNA as calibrator, each point of the dilution series being tested six times. The same measurements were performed with two reference genes located on chromosomes 15 (*B2M* gene) and 16 (*CNGB1* gene), because these two chromosomes are rarely modified in number in ALL,[21] using Ref15F and Ref15R primers for *B2M* and Ref16F and Ref16R primers for *CNGB1* (Supplementary Table S1). The percentage of each *PAX5* exon to the mean of Ref15 and Ref16 copy number was calculated. A value of 100% means that the number of *PAX5* copies is identical to that of the references, and corresponds to two copies.

PAX5 *point mutations*

PAX5 DNA mutation screening was performed by sequence analysis on both strands (*PAX5* exons 2, 3, 7, 8 and 9) and high-resolution melting PCR (HRM-PCR) for the remaining exons (Supplementary Table S1). Sequencing was performed using BigDye dideoxynucleotides and the products were separated on a 3130 XL sequencing apparatus (Applied Biosystems). Electrophoregrams were analyzed using the Sequencher software (version 4.1.2; Gene Codes Corporation, Ann Arbor, MI, USA) with a secondary peak threshold of 20% followed by manual verification. The presence of polymorphisms was evaluated using dbSNP and remission genomic DNA when available. HRM-PCR was performed using 1 × LightCycler 480 HRM Master Mix (Roche Applied Science, Mannheim, Germany) with 10 ng of genomic DNA, primers 0.1 μM and 25 mM $MgCl_2$. HRM-PCR cycling conditions were initial denaturation at 95 °C during 10 min followed by 50 cycles at 95 °C for 10 s, at 63 °C for 15 s and at 72 °C for 25 s. Melting curve was measured from 70 to 95 °C with 25 acquisitions per °C.

PAX5 *partial deletions*

Full-length cDNA amplification of partial deletion mutants was performed by PCR on a 2720 Thermal Cycler (Applied Biosystems) with the Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA). The amplification program was 5 cycles at 72 °C, 5 cycles at 70 °C and 25 cycles at 68 °C. Primers used were PAXQ and PCR5 (Supplementary Table S1). Reverse transcriptase (RT)–PCR products were analyzed by electrophoresis on an agarose gel, cut out and sequenced.

PAX5 *FISH analysis*

Fluorescent *in situ* hybridization analyses were performed on cases presenting either a chromosomal 9p13 breakpoint or deletion using a *PAX5* commercial probe (*PAX5* FISH DNA Probe, Split Signal, Y5413; Dako, Carpinteria, CA, USA) and/or a combination of two fluorescent-labeled bacterial artificial chromosome clones RP11-243F8 hybridizing *PAX5* from exon 1 to 9 and RP11-344B23 hybridizing from exon 7 to 10 and to an extended telomeric region. The TCF3 split signal FISH probe (TCF3 FISH DNA Probe, Split Signal, Y5402; Dako) was also used.

IGH/TCR *rearrangements analysis*

IGH and *TCR* rearrangements were evaluated by the four centers performing the minimal residual disease follow-up of the clinical trial (Paris-Necker, Paris-Saint-Louis, Paris-Robert-Debré and Lille) according to the BIOMED-2 Concerted Action BMH4-CT98-3936-2 protocol.[22] Immunoglobulin heavy chain specific amplification of the proximal V_H6-1 segment rearrangement with J_H was performed according to the BIOMED-2

protocol,[22] using the V_H6 FR2 primer along with the J_H consensus primer (Supplementary Table S1).

Statistical analysis

Binary variables were compared with the two-sided Fisher's exact test. The Mann–Whitney test was used for median comparisons. The median follow-up of surviving patients for the entire protocol was 37 months. Event-free survival (EFS) was calculated from the date of prephase initiation. Events accounting for EFS were failure of remission induction, relapse and death in first complete remission (CR). Failure time data were estimated by the Kaplan–Meier method,[23] then compared by the log-rank test.[24] Cumulative incidence estimations took into account competing risks and were compared by the Gray test.[25] A *P*-value less than 0.05 was considered to indicate statistical significance. All calculations were performed using the STATA/SE software, version 9.0 (Stata Corporation, College Station, TX, USA) and the R software, version 1.5.1 (The R Development Core Team, A Language and Environment Copyright, 2002).

Results

Representativeness of the samples tested

We screened the occurrence of *PAX5* mutations in a series of 117 BCP-ALL prospectively treated in the GRAALL-2003/GRAAPH-2003 study, with a median follow-up of 22 months (Table 1). The GRAALL-2003/GRAAPH-2003 included 300 adults with ALL. The 117 BCP-ALL cases analyzed for the occurrence of *PAX5* mutations were similar to the 107 other BCP-ALL cases of the trial for whom no material remained available for analysis. We confirmed the absence of difference regarding main patient characteristics, including age and WBC count, as well as corticosensitivity, chemosensitivity, first CR rate, overall survival and *BCR-ABL1* status (data not shown). Therefore, the population of 117 BCP-ALL analyzed for the presence of *PAX5* mutations is representative of the whole GRAALL-2003/GRAAPH-2003 protocol.

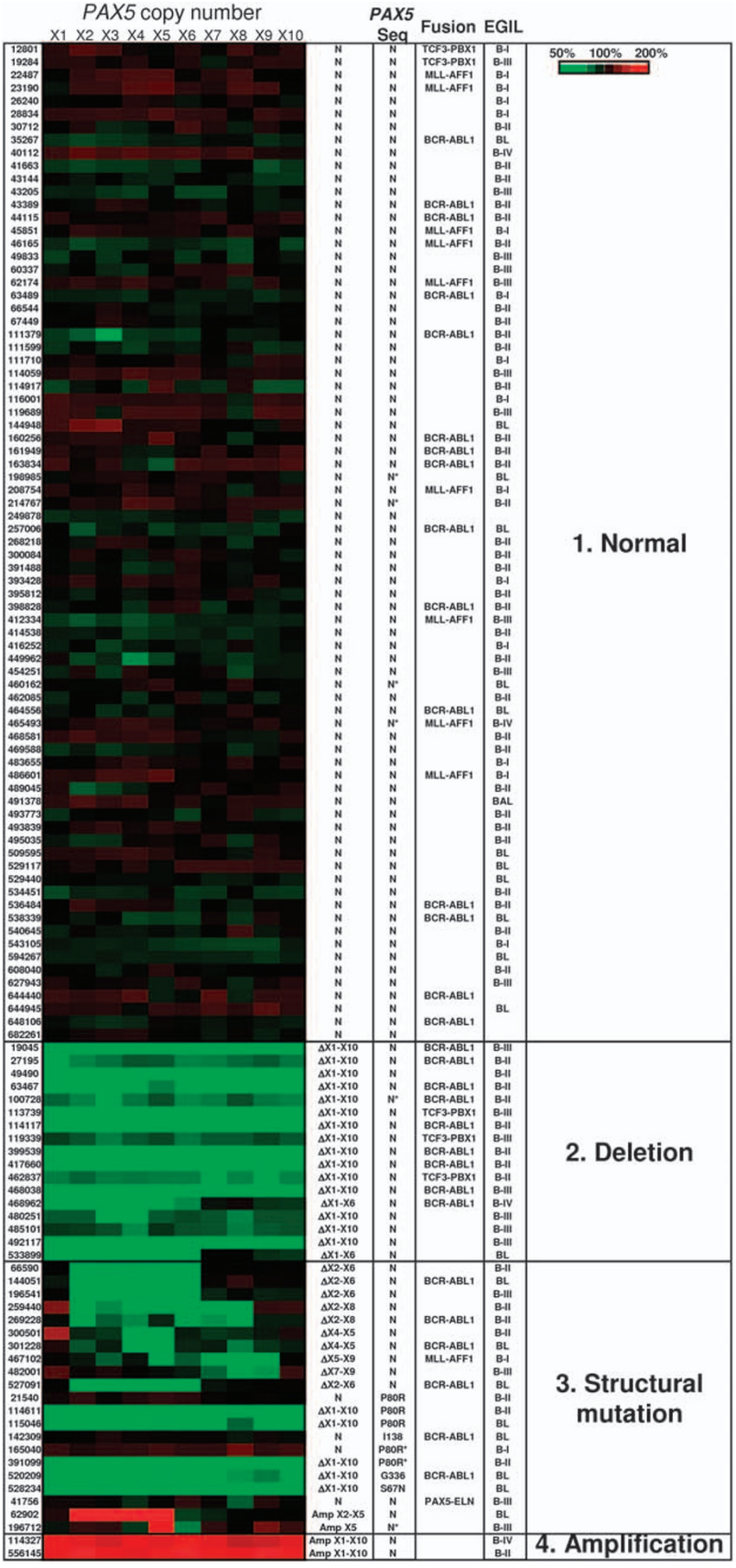
Genomic PAX5 *copy number*

Normal *PAX5* content was found in 81 patients (69%), 20 had a complete hemizygous deletion (17%), 12 a partial hemizygous deletion (10%), 2 a partial amplification (2%) and 2 a complete amplification (2%) (Figure 1; Supplementary Table S2). *PAX5* copy number results were validated by FISH analysis (Supplementary Table S3), karyotype (Supplementary Table S4) and by sequencing the partially deleted cDNA (Figure 2). These results indicate that alteration of *PAX5* exon copy number is a frequent event in adult BCP-ALL occurring in 31% of the cases.

Table 1 Statistical analysis of clinical and biological data

	Normal		*Deletion*		*Mutation*		*Pearson's* χ^2*-test*
Patients (*n*)	77		17		21		
Median age (years)	35 (17–60)		39 (16–55)		36.5 (16–60)		NS
Median WBC (10^9 per liter)	13.8 (0.9–348)		3.35 (1.2–78)		11.4 (1.2–343)		0.01
CR	68/76	89%	16/17	94%	18/20	90%	0.03
Relapse	19/75	25%	4/17	24%	8/20	40%	NS
Corticosensitivity	56/74	76%	14/17	82%	16/20	80%	NS
Chemosensitivity	32/69	46%	9/17	53%	9/20	45%	NS
CD19, CD10 and CD20 antigens							
CD19 positive	71/73	97%	17/17	100%	20/21	95%	NS
CD10 positive	50/71	70%	17/17	100%	19/21	90%	0.018
CD20 positive	24/70	34%	8/16	50%	10/20	50%	NS
EGIL classification							
B-I	15/73	21%	0/17	0%	2/21	10%	
B-II	33/73	45%	8/17	47%	7/21	33%	
B-III	10/73	14%	7/17	41%	4/21	19%	0.002
B-IV	2/73	3%	1/17	6%	0/21	0%	
BL	13/73	18%	1/17	6%	8/21	38%	
Cytogenetics/Fusion genes							
BCR-ABL1	15/77	19%	9/17	53%	6/21	29%	
TCF3-PBX1	2/77	3%	3/17	18%	0/21	0%	0.009
MLL rearrangement	10/77	13%	0/17	0%	1/21	5%	
del9p	2/62	3%	4/14	29%	3/20	15%	0.021
IGH/TCR rearrangements							
IGH VDJ positive	42/55	76%	10/11	91%	9/13	69%	NS
TCRG positive	28/54	52%	6/10	60%	10/13	77%	NS
TCRD positive	18/51	35%	4/10	40%	6/13	46%	NS

Abbreviations: BL, B-cell lineage; CR, first complete remission; EGIL, European Group for the Immunological Characterization of Leukemias; NS, not significant; WBC, white blood cell.
Clinical data were analyzed in the three groups (normal, deletion and structural mutant). Two patients with *PAX5* amplification are not included in the analysis. Main clinical features were analyzed including age, WBC, CR at the first attempt (CR), relapse after CR1, corticosensitivity and chemosensitivity. Differentiation block stages were analyzed using B-specific cell markers (CD19, CD10 and CD20) and EGIL classification (B-I, B-II, B-III, B-IV, BAL). *BCR-ABL1* and *TCF3-PBX1* fusion genes were significantly associated with the *PAX5* deletion group. No difference was found between the normal and structural mutant *PAX5* groups. Finally, *IGH* VDJ rearrangements and illegitimate *TCR* rearrangements were equally frequent between the three groups. Statistical *P*-value is indicated in the last column.


PAX5 copy number
X1 X2 X3 X4 X5 X6 X7 X8 X9 X10
PAX5 Seq
Fusion
EGIL
50% 100% 200%
1. Normal
2. Deletion
3. Structural mutation
4. Amplification

PAX5 *genomic deletion as a secondary event*

In two cases (patients 462837 and 119339), blasts carrying the *TCF3-PBX1* fusion gene and deletion of one *PAX5* allele were further analyzed using the relevant FISH probes together on the same slide. *TCF3* rearrangement was detected in 90% (patient 462837) and 80% (patient 119339) of the nuclei. Of these *TCF3*-rearranged nuclei, only 60% (patient 462837) and 20% (patient 119339) of the nuclei, respectively, had a *PAX5* deletion suggesting that, at least in these cases, *PAX5* deletion is a secondary event (Supplementary Figure S1).

PAX5 *point mutations*

PAX5 point mutations were investigated by sequence and HRM-PCR analyses in the same 117 BCP-ALL samples (except for 8 patients for whom no material remained for HRM-PCR analysis). Eight mutations were identified among the 117 cases thus representing 7% of the cases (Supplementary Figures S2 and S3). Among them, the P80R was the most frequent point mutation, occurring in five out of eight cases (patients 21540, 114611, 115046, 165040 and 391099). The three other mutations were one point mutation S67N (patient 528234) and two frameshift mutations (insertion of one G at the base 2 of exon 4 (codon I138, patient 142309) and insertion of two G at the base 97 of exon 8 (codon G336, patient 520209)). Five point mutations were associated with a complete deletion of the second *PAX5* allele (patients 114611, 115046, 391099, 520209 and 528234) (Figure 1).

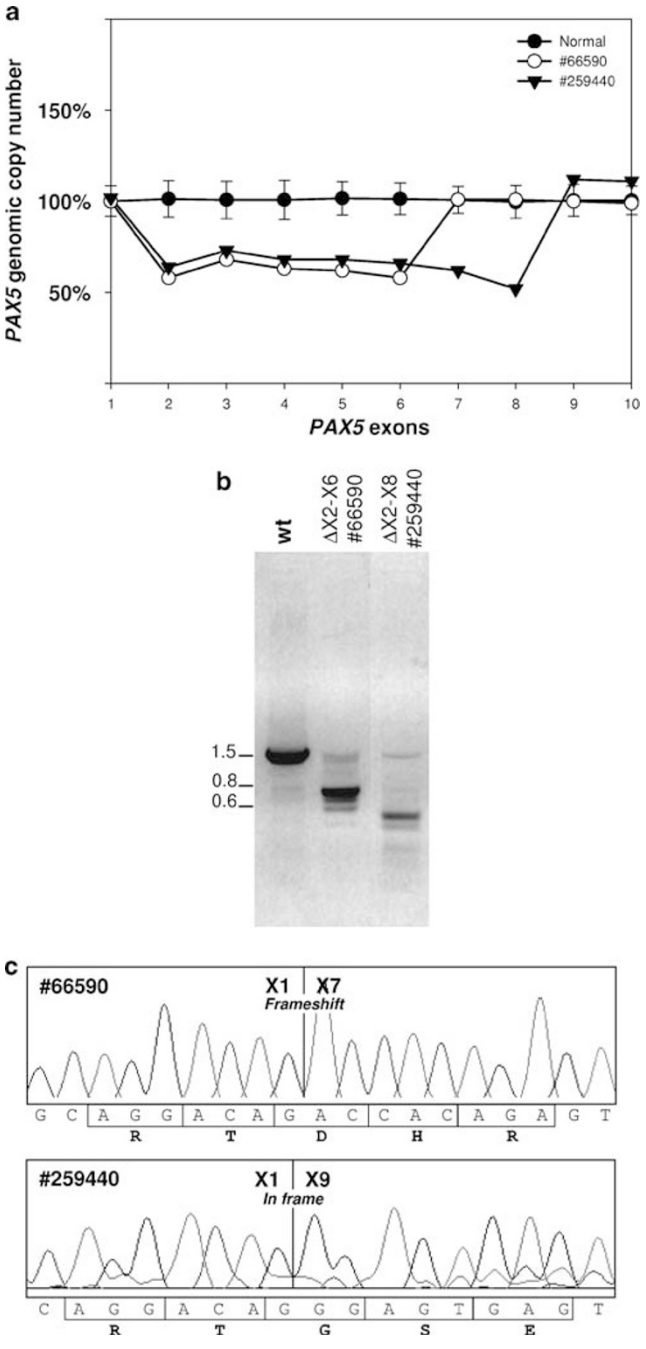


Figure 2 Validation of *PAX5* (paired-box domain 5) exon copy number quantification. (**a**) *PAX5* copy number quantification using quantitative PCR, 100% as two copies of a *PAX5* exon. Standard deviations calculated on the normal *PAX5* group are shown. Partial deletion of patients 66590 and 259440 are indicated. (**b**) *PAX5* mRNA expression of patients 66590 and 259440 showing a partial deletion of *PAX5*. cDNA were amplified by reverse transcriptase (RT)–PCR between *PAX5* exons 1 and 10. PCR products were analyzed on agarose gel. Lane 1 shows amplification of a 1505 bp normal RT–PCR product corresponding to the normal *PAX5* mRNA, lane 2 shows a smaller 770 bp product corresponding to a *PAX5* product with deletion from exon 2 to 6 and lane 3 a smaller 538 bp product corresponding to a *PAX5* product with deletion from exon 2 to 8. (**c**) Sequence chromatographs of the amplified PCR products. The upper panel shows the direct fusion between exons 1 and 7 and the lower panel shows direct fusion between exons 1 and 9 confirming the deletion.

Figure 1 PAX5 (paired-box domain 5) mutations in adult B-cell progenitor acute lymphoblastic leukemia (BCP-ALL). Genomic DNA content for each of the 10 exons (X1–X10) was determined by quantitative PCR for each patient (GRAALL-2003/GRAAPH-2003 identification number given in the first column). Results (detailed in Supplementary Table S2) are expressed using color code (first frame): less than 100% of the control in green (deletion), 100% ($=2n$ copies) in black (normal) and more than 100% in red (amplification). Patients were classified into four groups according to the *PAX5* status: normal *PAX5*, patients with two *PAX5* copies for each of the 10 exons; deleted *PAX5*, complete loss of one of the two *PAX5* alleles or deletion from *PAX5* exon 1; structural mutant *PAX5*, patients with partial deletion, partial amplification, point mutation or fusion gene, associated in some cases with deletion of the second allele; amplified PAX5, patients with a global amplification of *PAX5*. The second frame detailed the copy-number alterations. The third frame shows point mutations, N as normal. A star indicates when *PAX5* exons 1, 4, 5, 6 and 10 were not investigated by high-resolution melting (HRM) analysis due to absence of remaining material. The fourth frame indicates fusion genes identified. The fifth frame provides the European Group for the Immunological Characterization of Leukemias (EGIL) status for each patient (BAL, bi-lineage acute leukemia; BL, B-cell lineage).

PAX5 *chromosomal rearrangement*

Fluorescent *in situ* hybridization analysis was performed when a chromosomal abnormality involving the 9p chromosomal band was identified by standard karyotype. Eleven patients showed a rearrangement of the 9p13 chromosomal band (patients 49490, 49833; 41756; 62902; 66590; 114117, 144051, 144948, 196712, 301228 and 391099) and in a single case (patient 41756: 46,XY,t(7;9)(q21;p21)[17]) a translocation which fused *PAX5* and *ELN* without loss of the remaining normal allele, was detected.[16] Thus *PAX5* translocations occur in adult BCP-ALL but are a rare event (<1%).

PAX5 *alterations in BCP-ALL*

Globally, *PAX5* alterations were identified in 40 cases out of 117 adult BCP-ALL (34%). These alterations are heterogeneous consisting in complete loss in 17%, focal deletions in 10%, point mutations in 7% and translocations in 1% of the cases. To further analyze these mutations, we apportioned the patients according to the probable consequences of mutations (Figure 1). The *PAX5* mutated BCP-ALL were subdivided in three groups. A first group called deleted PAX5, pooling 17 cases (15%), characterized by a probable lower expression of the normal PAX5 protein due to a complete deletion of one *PAX5* allele or a partial deletion removing *PAX5* exon 1 with no mutation of the second allele. A second group called structural mutant PAX5 bringing together 21 cases (18%) characterized by the expression of a *PAX5* mutant allele, that is, having an altered activity regarding its transcriptional function consisting either of *PAX5* partial deletion conserving *PAX5* exon 1 (10 cases), partial amplification (2 cases), point mutations (8 cases) and fusion gene (1 *PAX5-ELN* case). A third group called amplified *PAX5* consisting of two cases (2%) that could lead to a higher expression of the normal PAX5 protein as a consequence of a whole *PAX5* genomic amplification. Owing to the small number of cases, this group was not further analyzed.

Characteristics of adult PAX5 *mutants BCP-ALL*

We compared the two main groups of *PAX5* mutations (deleted *PAX5* and structural mutant PAX5) to the largest category composed of patients without evidence of *PAX5* mutation (77 cases, 66%, labeled as normal *PAX5*).

Immunophenotype

We analyzed the membrane expression of major B-cell differentiation markers. We first analyzed CD19 as it is a direct target of PAX5.[26] However, no difference according to the *PAX5* status was observed (Table 1). CD20-positive cells were not significantly different in deleted *PAX5* (8 of 16, 50%), structural mutant *PAX5* (10 of 20, 50%) and normal *PAX5* groups (24 of 70, 34%) (Table 1). In contrast, no CD10-negative cases were detected in the deleted *PAX5* group (0 of 17) compared to 21 of the 71 (30%) normal *PAX5* cases ($P=0.018$) (Table 1). This difference is probably related to the fact that CD10-negative BCP-ALL are associated with *MLL* translocations[27] and *PAX5* alterations are not associated with *MLL* translocations (to the unique exception of patient 467102) The structural mutant *PAX5* group showed an intermediate result because 2 of 21 BCP-ALL (10%) lacked CD10 surface expression.

European Group for the Immunological Characterization of Leukemias (EGIL) classification allows a subdivision of BCP-ALL according to the stage of maturation block of the leukemic cells.[28] B-I represents the earliest stage and B-IV the latest stage of differentiation of BCP-ALL. Because the EGIL classification used the detection of CD10, B-I being CD10-negative BCP-ALL and B-II to B-IV being CD10 positive, we found an imbalanced repartition in EGIL subset between deleted and normal *PAX5* groups ($P=0.002$; Table 1).

Cytogenetics and fusion genes

Fusion gene rearrangements are frequent in BCP-ALL, especially *BCR-ABL1* in adult BCP-ALL. We evaluated the association of these fusion genes or other karyotypic features regarding *PAX5* status (Tables 1 and 2). We found a significant association between the presence of *BCR-ABL1* (53%) or *TCF3-PBX1* (18%) and the deleted *PAX5* group compared to the normal group (19% *BCR-ABL1* and 3% *TCF3-PBX1*) or the structural mutant PAX5 group (29% *BCR-ABL1* and 0% *TCF3-PBX1*) ($P=0.009$; Table 1). No *MLL* rearrangement and no normal karyotype were detected in the deleted *PAX5* group as compared to 10 (13%) and 20 cases (26%), respectively, in the normal *PAX5* group.

Table 2 Cytogenetic data

Groups	*Normal PAX5 (%)*		*Deletion PAX5 (%)*		*Mutant PAX5 (%)*		*Amplification PAX5 (%)*		*Total (%)*	
BCR-ABL1	15	19	9	53	6	29	0	0	30	26
MLL	10	13	0	0	1	5	0	0	11	9
TCF3-PBX1	3	4	3	18	0	0	0	0	6	5
47–50	8	10	0	0	0	0	0	0	8	7
51–65	2	3	1	6	1	5	0	0	4	3
Hypo/Triploid	0	0	0	0	0	0	1	50	1	1
41–45	0	0	1	6	0	0	0	0	1	1
Complex	5	6	1	6	2	10	1	50	9	8
Other	8	10	1	6	6	29	0	0	15	13
Normal	20	26	0	0	4	19	0	0	24	21
Failure	5	6	1	6	1	5	0	0	7	6
NA	1	1	0	0	0	0	0	0	1	1
	77		17		21		2		117	

Cytogenetic data: Cytogenetic features of GRAALL-2003/GRAAPH-2003 patients included in this analysis. BCR-ABL1: t(9;22) and/or BCR-ABL1-positive; MLL: MLL rearrangement; TCF3-PBX1: t(1;19) and/or TCF3-PBX1-positive; 47–50: low hyperdiploidy 47–50 chromosomes; 51–65: high hyperdiploidy 51–65 chromosomes; Hypo/Triploid: hypodiploidy 30–40 chromosomes and/or paratriploidy 60–80 chromosomes; 41–45: hypodiploidy 41–45 chromosomes; Complex: 5 or more chromosomal abnormalities; Other: chromosomal abnormality not otherwise classified; Normal: no cytogenetic/molecular abnormality detected; Failure: karyotype failure and no molecular abnormality; NA: not available.

IGH $V_HD_HJ_H$ and TCR rearrangement

Because in mice *Pax5* homozygous deletion impairs B-cell transition from D_HJ_H to $V_HD_HJ_H$ *IGH* rearrangement,[5] we investigated the rearrangement status of *IGH* in the context of *PAX5* mutational status. Complete $V_HD_HJ_H$ *IGH* rearrangements were detected in 76% (42 of 55), 91% (10 of 11) and 69% (9 of 13) of normal, deleted and mutant *PAX5* subgroups respectively. These differences were not statistically significant (Table 1). Although $V_HD_HJ_H$ rearrangement occurs, V_H segment proximal vs distal accessibility could be impaired in a similar way to the *IL7R* homozygous deletion model.[29] $V_HD_HJ_H$ rearrangements were sequenced in a unique center (Paris-Necker) in 19 BCP-ALL (12 normal *PAX5*, 3 deleted *PAX5* and 4 structural mutant PAX5). Intriguingly, the two PAX5 P80R mutants analyzed showed a rearrangement involving the proximal V_H6-1 segment, the closest V_H segment to J_H segments (only 1 of 12 in normal *PAX5* BCP-ALL). This V_H segment is usually rarely used in physiological situations and this bias suggests a possible impairment of accessibility to the *IGH* locus with this PAX5 P80R mutant. We therefore analyzed the use of the V_H6-1 segment and found that P80R (two of three analyzed) and S67N mutants used this V_H6-1 (data not shown). We also investigated the occurrence of illegitimate rearrangements of the *TCRG* and *TCRD* loci but no differences were detected according to *PAX5* mutational status.

Prognostic significance

There was no difference in median age between the normal, deleted and mutant *PAX5* subgroups (Supplementary Tables 1 and S5). Of note, median WBC was significantly lower in the deleted *PAX5* subgroups as compared to the two other subgroups (3.4 vs 13.8 (normal) and 11.4 G/L (structural mutations); $P=0.01$; Table 1).

Two patients eventually were not treated according to the protocol and the two patients showing an amplification of *PAX5* were not included in this analysis. In the 113 remaining patients, CR rate was 68 of 76 (89%), 16 of 17 (94%) and 18 of 20 (90%) in the normal, deleted and structural mutant PAX5 subgroups respectively ($P=0.03$; Table 1). The percentage of corticoresistant leukemia was 24, 18 and 20%, respectively, in these three subgroups (not significant; Table 1). A total of 42 patients were allografted in first CR (28 from the normal PAX5, 5 from the deleted *PAX5* and 9 from the structural mutant PAX5 subgroup, respectively). Cumulative incidence of relapse and DFS are shown in Figure 3, according to the *PAX5* status. As indicated, there were no significant differences in outcome among the three subsets (Figure 3a). However, it is worth noting that a trend toward a higher incidence of relapse in the structural mutant PAX5 subgroup was observed (Figure 3b). Results were similar when patients allografted in first CR were censored at transplant time (not shown). Of note, patients with *BCR-ABL1*-positive ALL (treated here with imatinib combined to conventional chemotherapy) displayed a similar outcome to those with *BCR-ABL1*-negative ALL in this series (not shown).

Discussion

We report here that *PAX5* is frequently mutated in adult BCP-ALL. *PAX5* mutations have been extensively reported in pediatric BCP-ALL.[12] In adult BCP-ALL so far, only *PAX5* deletion has been reported in small series of 26 adult and adolescent BCP-ALL[30] and 22 BCR-ABL1 positive adult BCP-ALL.[31] We investigated in this study, *PAX5* deletions but also *PAX5* structural mutations in a large series of 117 adult BCP-ALL in a unique clinical protocol. Out of the patients analyzed from the GRAALL-2003/GRAAPH-2003 clinical trial, 40 carried a mutation of the *PAX5* gene (34%). *PAX5* mutations occur in different ways, as complete or partial deletions, complete or partial amplifications, point mutations or fusion genes.[12] We analyzed *PAX5* mutants according to their probable functional consequences according to the study by Mullighan *et al*.[12] Isolated complete deletion of one *PAX5* allele is found in 15% of adult BCP-ALL (deleted *PAX5* group) and 11% of child BCP-ALL.[12] Mutant alleles are found in 18% of adult BCP-ALL (mutant PAX5 group) and 22% of children BCP-ALL.[12] PAX5 point mutations are detected in 7% of adult BCP-ALL and 7% of child BCP-ALL.[12] PAX5 P80R mutation is a frequent event in BCP-ALL detected in 5 of 117 cases in adult BCP-ALL and 4 of 192 cases in child[12] occurring therefore in 3% of BCP-ALL. We identified three new mutations such as PAX5 S67N, one G insertion in codon 138 (exon 4) and one insertion of two G in codon 336 (exon 8) leading both to a frameshift of *PAX5* last exons encoding the transactivation and inhibitory domains. Therefore, *PAX5* mutations appear to be similar, in nature and frequency, between child and adult BCP-ALL.

PAX5 maintains the identity of B cells.[7] Its homozygous deletion in mice blocks B-cell differentiation at an early stage.[5,6] In our series, out of the 17 cases of hemizygous deletion (deleted *PAX5* group), no early stage CD10-negative BCP-ALL were identified in contrast to the 15 of 74 cases (20%) found in the normal *PAX5* group. The proportion of these BCP-ALLs in the *PAX5* structural mutant group was intermediate between normal *PAX5* and deleted *PAX5* (2 of 21 cases, 10%). This result suggests that the hemizygous deletion, that is, a lower dose of normal PAX5, is either a later event or blocks the leukemic cells at a later stage during B-cell differentiation. An alternative explanation is linked to the absence of *PAX5* deletions in *MLL*-rearranged cases, and consequently the absence of CD10-negative BCP-ALL, the hallmark of MLL rearrangements,[27] is merely a consequence of this exclusion.

Fusion genes *BCR-ABL1*, *TCF3-PBX1* or involving *MLL* are frequently detected in adult BCP-ALL. We identified these fusion genes in 26, 4 and 9% of our cases respectively. *PAX5* deletions are highly skewed toward *BCR-ABL1* and *TCF3-PBX1* fusion genes, occurring in 71% of patients in deleted *PAX5* compared to 22% in normal *PAX5* and 29% in mutant *PAX5*, suggesting a very important role of PAX5 dosage during the transformation process of these two oncogenes. It is of note that the frequency of *PAX5* deletions is similar in child and adult BCP-ALL, even if associated events such as *BCR-ABL1* are not similarly distributed. In addition, *PAX5* deletion seemed to be a secondary event as suggested by our FISH analysis performed on blasts carrying both *TCF3-PBX1* fusion gene and *PAX5* deletion.

Structural mutations are heterogeneous, consisting of partial deletions, partial amplifications, point mutations and fusion genes. The complete deletion of one *PAX5* allele is frequently associated with deletion of *CDKN2A* located telomeric of *PAX5* in the short arm of chromosome. Transduction using a *BCR-ABL1* retrovirus in bone marrow cells of mice with only one copy of *PAX5* shorten drastically the survival (median from 60 to 36 days).[32] Moreover the concomitant haploinsufficiencies of *PAX5* and *CDKN2A* reduce again significantly the survival to a median of 21 days.[32] This demonstrates that loss of one copy of *CDKN2A* and *PAX5* is synergistic during the BCR-ABL1 transformation. PAX5 point mutations, such as P80R, and *PAX5* fusion genes are isolated events, not associated with BCR-ABL1. Partial deletion mutants target either the DNA-

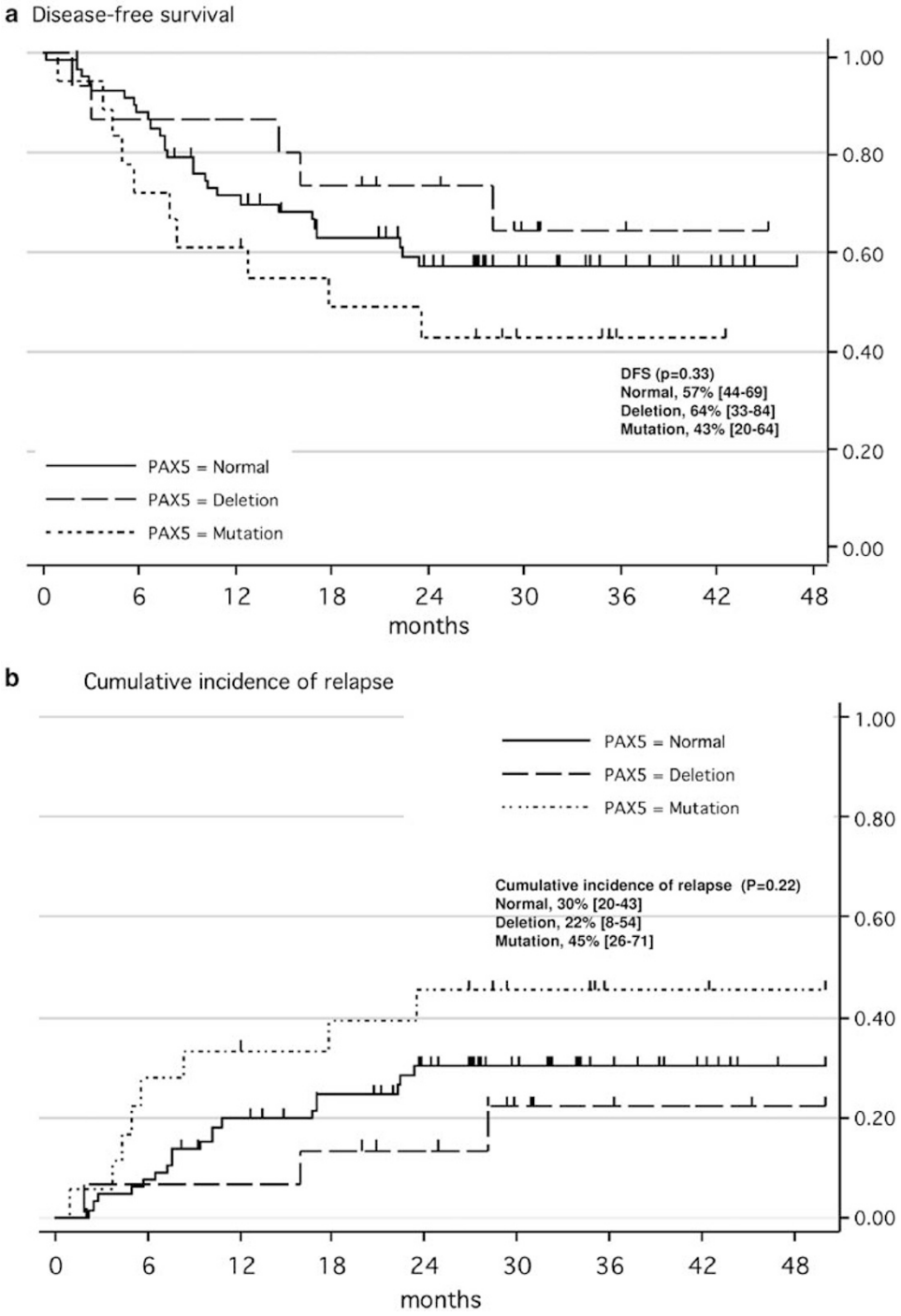


Figure 3 Disease-free survival (DFS) and cumulative incidence of relapse according to *PAX5* (paired-box domain 5) status (**a**) DFS. At 3 years, estimated DFS was 57% (95%; CI 44–69%), 64% (95%; CI 38–84%) and 43% (95%; CI 20–64%), in the normal, deleted and structural mutant *PAX5* group, respectively ($P=0.33$, by the log-rank test). (**b**) Cumulative incidence of relapse. At 3 years, estimated cumulative incidence of relapse was 29, 22 and 48%, in the normal, deleted and structural mutant PAX5 subgroup, respectively ($P=0.22$, by the Gray test).

binding domain or the transactivation domain. Deletion of the DNA-binding domain only is associated with BCR-ABL1 (two of the four cases). Frameshift structural mutants (I138 and G336) are also associated with BCR-ABL1 (for the two cases).

PAX5 point mutations are frequently associated with the deletion of the remaining allele (10 of 13 pediatric cases;[12] 5 of 8 cases in our series) suggesting that the mechanism of action of these point mutations during the oncogenic process may not be associated to a dominant-negative effect. By contrast, a dominant-negative action was established for PAX5-ELN,[16] PAX5-ETV6[11,12] and PAX5-FOXP1[12] fusions.

It has been reported that the most proximal V_H segment, V_H6-1, is overused in adult B-ALL patients.[33] Furthermore, *Pax5* homozygous deletion in mice showed that Pax5 is crucial for the transition from D_HJ_H to $V_HD_HJ_H$ *IGH* rearrangement, and especially for distal rearrangement.[34] Although we were unable to detect a significant frequency difference regarding *IGH*

$V_HD_HJ_H$ rearrangement between each group of BCP-ALL, we have therefore looked at the rearrangement at V_H6-1. Our results confirm that in average 11.4% (12 of 105) of the adult BCP-ALL tested overuse the V_H6-1 segment, suggesting a modification of the accessibility of the other V_H segments in this pathology, with a bias toward PAX5 DNA-binding mutants such as S67N or P80R.

In conclusion, *PAX5* mutations are frequent in adult BCP-ALL in accordance with the pediatric BCP-ALL cases.[12] Our data clearly show a difference regarding the type of *PAX5* mutations (deleted *PAX5* or structural mutant *PAX5*) in term of association to fusion genes, EGIL classification and WBC counts. The loss of an entire allele of *PAX5* seems to be a rather late event and might be considered as a secondary event.

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