Mutations in TP53 and JAK2 are independent prognostic biomarkers in B-cell precursor acute lymphoblastic leukaemia

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Background: In B-cell precursor acute lymphoblastic leukaemia (B-ALL), the identification of additional genetic alterations associated with poor prognosis is still of importance. We determined the frequency and prognostic impact of somatic mutations in children and adult cases with B-ALL treated with Spanish PETHEMA and SEHOP protocols.

Methods: Mutational status of hotspot regions of TP53, JAK2, PAX5, LEF1, CRLF2 and IL7R genes was determined by next-generation deep sequencing in 340 B-ALL patients (211 children and 129 adults). The associations between mutation status and clinicopathological features at the time of diagnosis, treatment outcome and survival were assessed. Univariate and multivariate survival analyses were performed to identify independent prognostic factors associated with overall survival (OS), event-free survival (EFS) and relapse rate (RR).

Results: A mutation rate of 12.4% was identified. The frequency of adult mutations was higher (20.2% vs 7.6%, P = 0.001). TP53 was the most frequently mutated gene (4.1%), followed by JAK2 (3.8%), CRLF2 (2.9%), PAX5 (2.4%), LEF1 (0.6%) and IL7R (0.3%). All mutations were observed in B-ALL without ETV6-RUNX1 fusions (P = 0.047) or BCR-ABL1 fusions (P < 0.0001). In children, TP53mut was associated with lower OS (5-year OS: 50% vs 86%, P = 0.002) and EFS rates (5-year EFS: 50% vs 78.3%, P = 0.009) and higher RR (5-year RR: 33.3% vs 18.6%, P = 0.037), and was independently associated with higher RR (hazard ratio (HR) = 4.5, P = 0.04). In adults, TP53mut was associated with a lower OS (5-year OS: 0% vs 43.3%, P = 0.019) and a higher RR (5-year RR: 100% vs 61.4%, P = 0.029), whereas JAK2mut was associated with a lower EFS (5-year EFS: 0% vs 30.6%, P = 0.035) and a higher RR (5-year RR: 100% vs 60.4%, P = 0.002). TP53mut was an independent risk factor for shorter OS (HR = 2.3; P = 0.035) and, together with JAK2mut, also were independent markers of poor prognosis for RR (TP53mut: HR = 5.9; P = 0.027 and JAK2mut: HR = 5.6; P = 0.036).

Conclusions: TP53mut and JAK2mut are potential biomarkers associated with poor prognosis in B-ALL patients.

B-cell precursor acute lymphoblastic leukaemia (B-ALL) is a malignancy of lymphoid progenitor cells, characterised by large biological and clinical heterogeneity (Roberts and Mullighan, 2015). ALL is the most frequent childhood cancer and also accounts for ~25% of adult acute leukaemias (Bhojwani et al, 2015). High-risk B-ALL disease is more likely with adults than...
children, and the long-term disease-free adult survival rates are <40%, despite intensive chemotherapy and/or allogeic stem cell transplantation treatments. This is in stark contrast to pediatric ALL, in which refined treatment regimens have resulted in cure rates approaching 80% (Paulsson et al., 2008; Bhojwani et al., 2015). However, in spite of this high cure rate, some children with ALL have a poor outcome, whereby 15% of them die from ALL relapses (Gowda and Dovat, 2013).

To date, the prognosis of B-ALL patients has focused mainly on clinical, haematological and genetic factors, such as age, leucocyte count at diagnosis, percentage of blast in peripheral blood, immunophenotype, central nervous system involvement, cyto genetic and molecular alterations and the presence of minimal residual disease (MRD), the latter two characteristics being most strongly associated with prognosis (Israel et al., 2010; Pui et al., 2011; Salari et al., 2014; Schrappe, 2014). However, ~30% of pediatric and 50% of adult ALL patients lack defined genetic hallmarks of biological and clinical significance (Bungaro et al, 2009; Dawson et al., 2011; Forero-Castro et al., 2016a; Forero-Castro et al., 2016b). Somatic mutations are hallmarks of lymphoid malignancies, and each genetic subtype harbours hidden mutations that are strong independent predictors of outcome (Iacobucci et al., 2012).

In recent years, the use of next-generation sequencing (NGS) has greatly increased the ability to identify somatic mutations with clinical impact in both child and adult B-ALL. These mutations involved genes associated with RAS signalling (48%; e.g., NRAS, KRAS, PTPN11, FLT3, Braf and NFI), B-cell differentiation and development (18%; e.g., PAX5, IKZF1, EBFB1, VPREB1), JAK/STAT signalling (11%; e.g., JAK1, JAK2, IL7R and CRLF2), cell cycle regulation and tumour suppression (6%; e.g., TP53, RB1, CDKN2A/B, PTEN and BTG1), and non-canonical pathways (9%, e.g., ETV6, CREBBP and TBL1XR1) (Harrison, 2011; Mullighan, 2011; Pui et al., 2011; Roberts and Mullighan, 2011; Iacobucci et al., 2012; Loh and Mullighan, 2012; Gowda and Dovat, 2013; Inaba et al., 2013; Chiaretti et al., 2014a; Chiaretti et al., 2014b; Woo et al., 2014). However, the prognostic impact of these mutations as predictors of clinical course, outcome and response to therapy is still being explored.

Herein, the frequency and clinical relevance of somatic mutations within a selected custom panel of six genes, TP53, JAK2, IL7R, PAX5, LEF1 and CRLF2 exons, was examined. Targeted exonic regions with known mutational hotspots (Harrison, 2011; Iacobucci et al., 2012; Inaba et al., 2013; Roberts and Mullighan, 2015), were analysed by amplicon-based NGS in 340 B-ALL patients. We demonstrated that mutations in TP53 and JAK2 have a negative impact on the outcome of pediatric and adult patients. This supports their role as prognostic biomarkers, and suggests that if assessed at diagnosis, they might contribute to a better risk stratification of B-ALL patients.

**RESULTS**

**DNA isolation.** Amplicon-based NGS was performed on all 340 samples obtained from untreated patients at diagnosis. Genomic DNA was extracted from frozen bone marrow or fixed peripheral blood cell samples with the QiAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions.

**Next-generation amplicon deep-sequencing assay.** An amplicon-based NGS assay was applied, using Titanium amplicon chemistry on a 454-GS Junior DNA Sequencing Platform (454 Life Sciences, Branford, CT, USA). With this approach, preconfigured custom 96-well primer plates containing lyophilised primer pairs (Roche, Branford, CT, USA) were used to prepare the amplicon library following the IRON-II Study procedures from the European Leukaemia Network group (Kohlmann et al., 2011). Nineteen hotspot exons were amplified: TP53 (E4-E11), JAK2 (E12-E16), PAX5 (E2-E3), LEF1 (E2-E3), CRLF2 (E6) and IL7R (E5). The size of amplicons ranged from 304 to 431 bp including the adaptor sequences (see Supplementary Material File 1: Supplementary Table S1 for primer sequences). Information about amplicon library pooling, purification, emulsion PCR, sequencing, validation of variants, data processing and analysis, is presented in the Supplementary Material File 1.

**CRLF2 gene expression analysis.** In a subset of 97 B-ALL patients (81 children and 16 adults) the expression levels of CRLF2 were evaluated by quantitative RT-PCR assays. CRLF2-overexpressed samples were identified as previously described (Yoda et al., 2010) (see Supplementary Material file 1 for further details).

**Statistical methods.** Continuous variables were summarised as their median and range; categorical variables were described as the frequency and percentage of subjects in each category. Associations between the presence of mutations and the values of clinical parameters were investigated. Continuous variables were analysed by the non-parametric Mann–Whitney U-test. The χ² or Fisher’s exact tests were used to identify significant associations between dichotomous variables, as appropriate. All tests were two-sided and values of P<0.05 were considered to be significant. Kaplan–Meier analysis (log-rank test) was used to assess the relationship between mutations and overall survival (OS), event-free survival (EFS) and relapse rate (RR). Survival rates were given as probabilities of survival at 5 years, with a 95% confidence interval (CI). Pediatric and adult patients were analysed separately. Survival criteria are shown in the Supplementary Material File 1. Clinical and genetic variables were first analysed by univariate analysis, and those with a P-value up to 0.05 were included in multivariate analysis. A multivariate Cox proportional hazards regression model was used to estimate the hazard ratio (HR) and 95% CI of risk factors. Specifically simultaneous regression, which SPSS calls the Enter method was used. Analyses were performed using SPSS version 22.0 (IBM).

**Patient characteristics.** Table 1 shows the clinical characteristics of the pediatric and adult patients with B-ALL included in this study.
study. The median age was 12 years (range 0–84 years); childhood patients were aged from 0 to 17 years (median 5 years) while adult patients were aged from 18 to 84 years (median 45 years). The median percentage of blast counts in bone marrow was 90% (range, 35–100%) and 30.6% of patients showed normal cytogenetics. The presence of poor prognosis abnormalities (t(9;22) (Ph + ), t(v;11q23) and hyperdiploidy) were more frequent in adult than in childhood B-ALL (48.8% vs 8.1%, P < 0.0001). In addition, there was a higher prevalence of highly hyperdiploid cases (> 50 chromosomes) in the pediatric compared with the adult group (15.6% vs 3.9%, P = 0.001). The median follow-up of the whole series was 60 months (range, 2–186 months). The children had higher rates of 5-year OS (85.3% vs 40.5%, P < 0.0001), EFS (77.7% vs 28.6%, P < 0.0001) and relapse (RR) (16.1% vs 48.1%, P < 0.0001) compared with adults.

Frequency and characterisation of molecular mutations in B-ALL

Thirty different mutations were observed in 42 out of 430 patients at diagnosis (12.4%). TP53 was the most frequently mutated gene, being present in 41% of patients, followed by JAK2 (3.8%), CRLF2 (2.9%), PAX5 (2.4%), LEF1 (0.6%) and IL7R (0.3%). The mutations were more frequent in adults than in children (20.2% vs 7.6%, P = 0.001). The detailed frequency of mutations in children and adults was TP53 (2.4% vs 7.0%, P = 0.038), CRLF2 (0.5% vs 7.0%, P = 0.001), JAK2 (2.4% vs 6.2%, P = 0.086), PAX5 (1.4% vs 3.9%, P = 0.162), LEF1 (0.5% vs 0.8%, P = 1.0) and IL7R (0.5% vs 0%, P = 1.0) (Supplementary Material File 1: Supplementary Figures S1 and S2).

The clinical characteristics, cytogenetic subgroups, somatic mutations with their respective mutational burden, risk classification, frontline therapy protocol used, response to therapy, survival and clinical status of each pediatric and adult patient are summarised in Supplementary Material File 1: Supplementary Table S2. Most mutations (88.1%) were detected in B-ALL cases lacking recurrent fusion genes. In fact, the presence of mutations in patients with normal cytogenetics was more frequently observed in both childhood (37.5%) and adult (46.2%) B-ALL cohorts (P = 0.002) (Supplementary Material File 1: Supplementary Table S3). It should be noted that only one pediatric patient (ID10) with TP53mut harboured the TCF3(E2A)-PBX1 fusion and four patients (one child, ID13 and three adults, ID25, ID26 and ID34) with PAX5mut, TP53mut and/or CRLF2mut carried KMT2A(MLL)-R.

Supplementary Material File 1: Supplementary Table S4 details the frequency of secondary somatic mutations in hotspot regions of TP53, JAK2, PAX5, LEF1, CRLF2 and IL7R genes classified by primary chromosomal abnormalities in children and adult patients with B-ALL. In the entire childhood cohort of B-ALL, all 16 mutations were exclusively detected in the subgroup of patients without ETV6-RUNX1 (TEL-AML1) translocation (P = 0.047). Moreover, in the entire adult cohort of B-ALL, all 26 mutations were exclusively detected in the subgroup of patients without BCR-ABL1 translocation (P < 0.0001). Thus, none of the cases with secondary somatic mutations showed ETV6-RUNX1 (TEL-AML1) or BCR-ABL1 translocations as primary chromosomal abnormalities. There were no other correlations between the primary chromosomal abnormality and the spectrum of mutations observed.

Supplementary Material File 1: Supplementary Table S5 details the primary chromosomal abnormalities evaluated by conventional and molecular cytogenetics, and describes the secondary somatic mutations according to the number of mutations per case, their mutational burden, gene-exon mutated and type of mutation observed in children and adult patients with B-ALL. The median mutational burden was 24.5% (range, 2–97%) (16.5% children and 28% adults, P = 0.730). It is of particular note that 25.9% of mutations detected showed mutation loads of ≤10% (23.5% children and 27% adults, P = 1.0), so these variants would not have been detected by the Sanger capillary sequencing method (Supplementary Material File 1: Supplementary Table S2).

Twenty-one of the 30 different mutations found were previously described in the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and/or the IARC TP53 database (http://p53.iarc.fr/p53Sequences.aspx) (Leroy et al, 2014) but we identified eight undescrbed mutations in those genes (one in each gene under study and three novel missense mutations in PA5X). Supplementary Material File 1: Supplementary Table S6 showed the protein domain affected by these mutations. The sequence analysis revealed 24 missense mutations, three deletion-insertions, two splicing mutations and one frameshift mutation. As shown in Supplementary Material File 1: Supplementary Figures S1 and S2, TP53 mutations were generally distributed across several exons, with missense mutations being predominantly found in evolutionarily conserved regions of this gene. The other mutated genes showed recurrent mutations: CRLF2 (P.F232C, nine patients), JAK2 (p.R683G, nine patients and p.R683S, nine patients) and PA5X (P.P80R, four patients).

NGS enabled identification of the co-occurrence of mutations and prediction of the presence or absence of distinct subclones harbouring different mutations. Eleven out of the 42 patients with mutations (26.2%) concomitantly harboured more than one mutation, which were more frequently observed in adult than in pediatric patients (38.5% vs 6.3%, P = 0.030). Ten cases had two and one adult case had three mutations in the genes investigated. All of them were orthogonally validated by other methods such as Sanger sequencing, re-sequencing run and/or the IonTorrent sequencer system. Supplementary Material File 1: Supplementary Table S7 shows the distribution of mutations identified in these 11 patients and Supplementary Material File 1: Supplementary Table S2 details their clinical characteristics, frontline therapy and outcome. In four cases (child: ID5 and adults: ID40, ID26 and ID27), the mutations occurred in the same gene and in the same amplicon (JAK2, PAX5 or TP53), while one adult patient (ID39) showed two different mutations within two amplicons of the same gene (TP53). In five adult cases, mutations occurred in different genes: three patients (ID17, ID21 and ID42) harboured concomitant JAK2 and CRLF2 mutations, while one patient (ID25) had TP53/PAX5 and, other (ID19) CRLF2/LEF1 mutations. Finally, one adult patient (ID31) harboured three concomitant mutations in JAK2 and CRLF2, of which two mutations were detected in the same JAK2 amplicon.

The amplicon-NGS allowed separate subclones to be distinguished in two adult patients (ID31 and ID40, Supplementary Material File 1: Supplementary Figure S3 and Supplementary Table S2) with the same combination of JAK2 mutations located in exon 16 (JAK2-E16, p.R683G and p.R683S). In these cases the mutated codon 683 was not found concomitantly within the same sequencing read, but was separated across distinct individual reads. In accordance with the differential mutational loads between the two JAK2 mutations, the sequencing analysis demonstrated that the dominant clone harboured the p.R683G mutation (c.2047A>G), whereas the minority clone harboured the p.R683S mutation (c.2049A>T). In both cases, the c.2047A>G mutation was always present at a higher percentage with respect to the c.2049A>T mutation (patient ID31: 26% and 2%; and patient ID40: 38% and 4%) and the minority subclone was present with a mutational burden of <5% of leukaemic cells. Finally, one of these patients (patient ID31) harboured a third low-percentage mutation in the CRLF2 gene (p.F232C in 10% of leukaemic cells).

In addition, amplicon-NGS made it possible to identify clonal heterogeneity in three patients (one child and two adults) who carried a double mutation within the same amplicon. In the first
Table 1. Characteristics of pediatric and adult B-ALL patients included in the study

| Characteristics | Whole cohort | Children < 18 years | Adults ≥ 18 years | P |
|-----------------|-------------|---------------------|------------------|---|
| N               | %           | n                   | %                | %  | P     |
| All patients    | 340         | 100                 | 211              | 100 | 129   | 100 | NA   |
| Age at diagnosis (years), median (range) | 12          | (0–84)              | 5                | (0–17) | 45    | (18–84) | NA |
| Sex             |             |                     |                  |     |       |     |      |
| Male, n (%)     | 168         | 49.4                | 94               | 44.5 | 74    | 57.4 | 0.022 |
| Female, n (%)   | 172         | 50.6                | 117              | 55.5 | 55    | 42.6 |       |
| Counts and other parameters |             |                     |                  |     |       |     |      |
| Bone marrow blast*, median (range) | 90          | (35–100)            | 90               | (35–100) | 88    | (35–98) | 0.011 |
| WBC count (× 10^3/l−1), median (range) | 15          | (1–634)             | 15               | (1–634) | 25    | (1–575) | 0.011 |
| WBC ≥ 30 × 10^3/l−1, median (range) | 112         | 38.4                | 59               | 32.2 | 53    | 48.6 | 0.005 |
| Hb count (g/l−1), median (range) | 90          | (26–172)            | 76               | (26–144) | 103   | (39–172) | <0.0001 |
| Platelet count (× 10^9/l−1), median (range) | 53          | (2–580)             | 64               | (3–556) | 50    | (2–580) | 0.251 |
| Elevated LDH (U/l−1) levelb, n (%) | 204         | 85.7                | 122              | 84.1 | 82    | 88.2 | 0.386 |
| ECOG score > 2, n (%) | 22          | 33.3                | 5                | 23.8 | 17    | 37.8 | 0.262 |
| Down syndrome, n (%) | 8          | 2.4                 | 6                | 2.9  | 2     | 1.6  | 0.715 |
| Cytogenetics    |             |                     |                  |     |       |     |      |
| Normal, n (%)   | 104         | 30.6                | 68               | 32.2 | 36    | 27.9 | 0.401 |
| Abnormal, n (%) | 236         | 69.4                | 143              | 67.8 | 93    | 72.1 |       |
| Cytogenetic risk groups |             |                     |                  |     |       |     |      |
| Poor risk, n (%) | 80          | 23.5                | 17               | 8.1  | 63    | 48.8 | <0.0001 |
| Others, n (%)   | 260         | 76.5                | 194              | 91.9 | 66    | 51.2 |       |
| Risk groupsc    |             |                     |                  |     |       |     |      |
| Low risk, n (%) | 57          | 19.3                | 57               | 33.9 | 0     | 0    | <0.0001 |
| Standard (intermediate) risk, n (%) | 69         | 23.3                | 54               | 32.1 | 15    | 11.7 |       |
| High risk, n (%) | 170         | 57.4                | 57               | 33.9 | 113   | 88.3 |       |
| MRD at the end of induction* |             |                     |                  |     |       |     |      |
| MRD < 0.01%, n (%) | 165        | 69.6                | 119              | 73.5 | 46    | 61.3 | 0.059 |
| MRD ≥ 0.01%, n (%) | 72          | 30.4                | 41               | 26.5 | 29    | 38.7 |       |
| Outcome data   |             |                     |                  |     |       |     |      |
| SCT performed in first CR, n (%) | 48          | 68.6                | 21               | 67.7 | 27    | 69.2 | 0.894 |
| Relapse, n (%)  | 76          | 27.7                | 34               | 18.1 | 42    | 48.8 | <0.0001 |
| Very early relapse*, n (%) | 41          | 27.7                | 13               | 40.6 | 28    | 68.3 | 0.06  |
| Patients alive in first CR, n (%) | 174         | 57.4                | 144              | 84.2 | 30    | 31.6 | <0.0001 |
| Deaths, n (%)   | 107         | 33.5                | 32               | 16.3 | 75    | 61   | <0.0001 |
| Median follow-up (range), months | 60          | (2–166)             | 66               | (2–146) | 39    | (3–171) | 0.002 |
| S-year OS rates % (95% CI) | 68.8        | (NR, 60.0–71.5)    | 85.3             | (NR, 77.1–88.5) | 40.5  | (17, 9.1–24.8) | <0.0001 |
| S-year EFS rates % (95% CI) | 60.1        | (NR, 50.7–62.7)    | 77.7             | (NR, 81.1–86.8) | 28.6  | (10, 5.3–14.6) | <0.0001 |
| S-year RR % (95% CI) | 26.1        | (NR, 25.1–38.0)    | 16.1             | (NR, 13.2–26.0) | 48.1  | (70, 50.6–89.5) | <0.0001 |

Abbreviations: B-ALL = B-cell precursor acute lymphoblastic leukaemia; CI = confidence interval; CR = complete remission; ECOG = Eastern Cooperative Oncology Group; EFS = event-free survival; HR = hazards ratio; LDH = lactate dehydrogenase; MRD = minimal residual disease; NA = not applicable; NR = not reached; OS = overall survival; RR = relapse rate; SCT = stem cell transplantation; WBC = white blood cell.

Probabilities highlighted in bold indicate statistically significant results (P < 0.05).

*By flow cytometry.

+By Cytogenetic risk groups established according to PHEMA protocols, based on age, WBC and cytogenetic subgroup.

+Time of relapse criteria: very early, earlier than 18 months after initial diagnosis and less than 6 months after cessation of frontline treatment; early, more than 18 months after initial diagnosis, but less than 6 months after cessation of frontline treatment; late, more than 6 months after cessation of frontline treatment.

patient (ID27-adult) who harboured two distinct mutations in the same amplicon (PAX5-E03, c.215A > G and c.239C > G), 454 deep-sequencing allowed the presence of four lines to be discriminated; one minority and germline (PAX5wt: 8.8%, one dominant clone with c.215A > G mutation (51.4%), one subclone with c.239C > G mutation (36.6%) and one minority subclone (3.2%) with a double mutation in the PAX5-E03 gene (c.215A > G and c.239C > G) (Supplementary Material File 1: Supplementary Figure S4). In the second patient (ID5-child) who carried two mutations in the JAK2-E16 gene, three lines were identified: one germline (JAK2wt: 84%), one subclone with c.2044_2045insGGACCTCCTCCTGCCCTC mutation (12%) and one minority subclone with c.2049A > T mutation (4%) (Supplementary Material File 1: Supplementary Figure S5). Finally, three independent lines were identified in the third patient (ID26-adult) presenting two mutations in the TP53-E08 gene: one germline (TP53wt: 83.4%) line, and two minority lines, one subclone with c.841G > A mutation (10%) and one subclone with c.845G > C mutation (6.6%) (Supplementary Material File 1: Supplementary Figure S6).

Mutated genes associated with clinical and prognostic features. Next, mutations were associated with cytogenetic subtypes, clinical features and risk factors commonly used to stratify pediatric and adult B-ALL patients (Supplementary Material File 1: Supplementary Table S8 and Supplementary Table S9). Thus, the presence of mutations in any of the genes analysed was associated with children stratified in the high-risk group (P = 0.018). Mutations in the TP53 gene were associated with poor response to frontline therapy due to refractoriness or relapse events (P = 0.032) (Supplementary Material File 1: Supplementary Table S8). Meanwhile, in the adult cohort, all 26 mutated cases were exclusively detected in BCR-ABL1-negative cases (P < 0.0001). The presence of JAK2mut was associated with poor-prognosis.
Prognostic value of somatic mutations in B-ALL

Gene mutations are related to a worse outcome. In the survival analysis of the children, significantly lower OS (5-year OS: 50% vs 86%, \( P = 0.002 \)) and EFS rates (5-year EFS: 50% vs 78.3%, \( P = 0.009 \)) and higher RR (5-year RR: 33.3% vs 18.6% \( P = 0.037 \)), were observed in patients with \( TP53 \) mut compared with patients without \( TP53 \) mut (Figure 1 and Supplementary Material file 1: Supplementary Table S10). The present study also confirmed in pediatric cohort the well-known associations of particular clinical and biological variables with worse prognosis, such as the high-risk group (OS: \( P < 0.0001, \) EFS: \( P < 0.0001 \) and RR: \( P = 0.002 \)), MRD \( > 0.01 \% \) (OS: \( P = 0.004 \) and EFS: \( P = 0.027 \)), WBC \( \geq 30 \times 10^9 \) (OS: \( P = 0.004 \), poor-risk cytogenetics due to the presence of t(9;22), t(v;11q23) or a hypodiploid karyotype (OS: \( P < 0.0001 \), EFS: \( P < 0.0001 \) and RR: \( P = 0.016 \)), age over 10 years (OS: \( P < 0.0001, \) EFS: \( P < 0.0001 \) and RR: \( P < 0.0001 \)), and a Pro-B phenotype (OS: \( P < 0.0001 \) and EFS: \( P = 0.004 \)) (Supplementary Material file 1: Supplementary Table S10). Multivariate analysis of the group of children showed that \( TP53 \) mut was an independent risk factor associated with significantly shorter OS (HR = 4.5; 95% CI 1.1–19.2, \( P = 0.04 \)) (Table 2).

In the group of adults, \( TP53 \) mut had a negative effect on OS (5-year OS: 0% vs 43.3%, \( P = 0.019 \)) and was associated with a higher RR (5-year RR: 100.0% vs 61.4%, \( P = 0.029 \)), and \( JAK2 \) mut was related with a lower EFS (5-year EFS: 0% vs 30.6%, \( P = 0.035 \)) and a higher RR (5-year RR: 100% vs 60.4%, \( P = 0.002 \)) (Figure 2 and Supplementary Material File 1: Supplementary Table S11). The NGS analysis also confirmed the clinical parameters commonly associated with shorter OS, EFS and RR in adults, such as MRD \( \geq 0.01 \% \) (RR: \( P = 0.003 \) and EFS: \( P = 0.023 \)), WBC \( \geq 30 \times 10^9 \) (RR: \( P = 0.024 \) and EFS: \( P = 0.008 \)), poor-risk cytogenetic abnormalities (OS: \( P = 0.013 \) and EFS: \( P = 0.025 \)) and age \( > 55 \) years (OS: \( P = 0.001 \), RR: \( P = 0.027 \) and EFS: \( P = 0.002 \)). Supplementary Material File 1: Supplementary Table S11 shows these clinical parameters commonly associated with shorter survival in adults. Multivariate analysis of the whole cohort of ALL adults indicated that the presence of \( TP53 \) mut was an independent risk factor associated with significantly shorter OS (HR = 2.3; 95% CI 1.1–5.1, \( P = 0.035 \)). Moreover, \( TP53 \) mut (HR = 5.9; 95% CI 1.2–28.6, \( P = 0.027 \)) and \( JAK2 \) mut (HR = 5.6; 95% CI 1.1–28.1, \( P = 0.036 \)) retained their independent prognostic significance in multivariate analysis regarding for RR (Table 3).

Finally, it is worth mentioning that there were no associations between the mutational burden and the cytogenetic, clinical and prognostic parameters of the pediatric and adult cohorts with B-ALL. In the same way, the survival rates of patients who carried...
small clones with ≤10% of mutational burden did not have significant differences with respect to those who carried clone sizes >10% (children: 5-year OS: 50% vs 72.7%, P = 0.544; 5-year EFS: 50% vs 72.7%, P = 0.606 and 5-year RR: 50.0% vs 20%, P = 0.426; adults: 5-year OS: 0% vs 31.3%, P = 0.453 and 5-year EFS: 0% vs 12.5%, P = 0.710). Particularly, for TP53 and JAK2 mutations, there was no difference of mutational burden on outcome and survival parameters.

CRLF2 overexpression is associated with JAK mutations. Overexpression of the CRLF2 gene (CRLF2oe) was observed in 17.5% of B-ALL patients by quantitative RT-PCR assays. There were no significant differences in the CRLF2oe between child and adult B-ALL patients (17.3% vs 18.8%, P = 1.0). CRLF2oe was common only in B-ALL cases that lack rearrangements of TEL-AML1, KMT2A(MLL), TCF3(E2A)-PBX1, and BCR-ABL (P = 0.007). Supplementary Material File 1: Supplementary Table S2 shows the results obtained from the analysis of CRLF2 expression in each of the patients with mutations. The presence of JAK2 mutations was associated with CRLF2oe (P = 0.023). Furthermore, neither the clinical nor prognostic features were associated with CRLF2oe in both childhood and adult B-ALL patients. However, the survival analysis of whole cohort of adults with B-ALL showed that the presence of CRLF2oe had a negative effect on OS (5-year OS: 0% vs 53.8%, P = 0.005) and EFS rates (5-year EFS: 0% vs 33.3%, P = 0.006).

**DISCUSSION**

The presence of gene mutations is a hallmark of B-ALL. In this study, we evaluated 340 B-ALL cases at diagnosis (211 children and 129 adults) to address the incidence and prognostic impact of TP53, JAK2, IL7R, PAX5, LEF1 and CRLF2 mutations by NGS. The incidence (12.4%) and frequency of mutations were higher in adults than in children according with the better prognosis of pediatric B-ALL. Most mutations (88.1%) were detected in B-ALL cases lacking recurrent fusion genes. The incidence and frequency of mutations detected in our study were similar to observations made in previous ALL studies (Mullighan, 2011; Pui et al, 2011; Roberts and Mullighan, 2011; Iacobucci et al, 2012; Loh and Mullighan, 2012; Gowda and Dovat, 2013; Inaba et al, 2013; Chiaretti et al, 2014a, b; Woo et al, 2014). The use of the NGS strategy further allowed the identification of clonal heterogeneity in B-ALL patients. A negative impact of the presence of TP53 and JAK2 mutations on the OS, RR and EFS in patients with adult and childhood B-ALL was found in the present study. Therefore, deep sequencing may ultimately be a better guide to treatment decisions for B-ALL patients.

Among 30 mutations identified at diagnosis, 22 have previously been described in B-ALL databases (Bercovich et al, 2008; Mullighan et al, 2009b; Roll and Reuther, 2010; Yoda et al, 2010; Mullighan et al, 2011; Izraeli et al, 2014; Leroy et al, 2014), confirming these mutated exons to be hotspot regions in leukaemia. Recurrent mutations in CRLF2 (p.F232C), JAK2 (p.R683G and p.R683S) and PAX5 (p.P80R) have also been observed in other cohorts of patients with ALL (Bercovich et al, 2008; Mullighan et al, 2009b; Roll and Reuther, 2010; Yoda et al, 2010; Mullighan et al, 2011; Izraeli et al, 2014). It is of particular note that we have found five undescribed mutations in those genes, three of which are missense mutations in PAX5. Mutations in the paired box (PAX) domain are predicted as deleterious, probably impairing the DNA-binding capability of this lymphoid transcription factor which is essential for normal B-cell development (Roberts et al, 2001). Also, two undescribed mutations were found in either IL7R or LEF1, the least frequently mutated genes in our series. Somatic gain-of-function mutations in IL-7R have been

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Figure 2. Kaplan–Meier curves for OS, EFS and RR of the whole cohort of adults with B-ALL. (A and B) OS and RR in patients bearing TP53mut. (C and D) EFS and RR in patients with JAK2mut.
shown to act as oncogenes in T- and B-ALL (Shochat et al., 2011; Mazzucchelli et al., 2012). (Shochat et al., 2011). These IL-7R mutations are usually insertions of bases encoding a cysteine and a proline into the 6th exon of the extracellular domain, at the border with the transmembrane region, immediately before or after residue 244 (Mazzucchelli et al., 2012). It is well known that cysteines and prolines are essential for the constitutive activation of the receptor causing cytokine independent growth of mouse pro-B cells (Shochat et al., 2011).

In the case of TP53mut, 11 out of 13 patients were negative for fusion genes, consistent with the findings of a previous ALL cohort at diagnosis (Chiaretti et al., 2013). Only one pediatric patient (ID10) with the TCF3(E2A)-PRX1 fusion and one adult (ID26) with KMT2A(MLL)-R harboured TP53mut. It should be noted that a significant association between TP53mut and MLL/AF11 translocations has been reported (Hof et al., 2011). Furthermore, two patients with low hypodiploid (ID9 and ID38) and two patients with near triploid karyotypes (ID22 and ID24) carried TP53 mutations. The high incidence of TP53 alterations (mutation, deletion) was previously associated with low hypodiploid/near triploid ALL, making it possible to distinguish this rare subset from other ALL subgroups including near haploid ALL. Furthermore, this high incidence can guide the prognosis of the disease because these alterations are usually associated with worse prognosis (Mullicher et al., 2014; Stengel et al., 2014). Therefore, the use of NGS could enable the prognostic B-ALL subgroups of patients to be refined.

Amplcnc-based NGS was also able to detect variants with a low mutational burden, which have been identified in genomic databases (Bercovich et al., 2008; Mullighan et al., 2009b; Roll and Reuther, 2010; Yoda et al., 2010; Mullighan et al., 2011; Izraeli et al., 2014; Leroy et al., 2014) or found in previous studies in ALL patients (Shochat et al., 2011). Thus, our study confirmed that NGS is a suitable method for accurately detecting and quantifying a variety of mutations in important genes associated with pathogenesis and prognosis of B-ALL. In fact, 26.4% of variations described in our study had mutation loads of ≤10%, underlining the high sensitivity of amplicon-based NGS for detecting small leukaemic subclones, commonly undetectable by conventional Sanger capillary sequencing. It is well known that mutational screening by bidirectional Sanger sequencing does not reveal the presence of mutant subclones representing less than 10–20% of leukaemic cells (Kastner et al., 2014).

One of the most important tasks in understanding clonal progression in leukaemia is to assess the nature and number of different subclones within an individual cancer (Mullighan et al., 2008; Grossmann et al., 2011; Landau et al., 2014). In agreement with the findings of previous studies (Kohlmann et al., 2010; Grossmann et al., 2011; Kohlmann et al., 2011; Kastner et al., 2014; Landau et al., 2014), we confirmed the complex clonal architecture of ALL. NGS technology allowed the detection of clonal heterogeneity in some of the analysed patients. This was achieved by identifying distinct subpopulations with a dominant leukaemic clone and their relative proportions in the total B-ALL cell population. The case ID27 (Supplementary Material File 1: Supplementary Figure S4) shows the presence of leukaemia subclones that were derived from a common tumour-initiating cell, whereas cases ID5 and ID26 (Supplementary Material File 1: Supplementary Figures S5 and S6) have clones with divergent mutations (Jan and Majeti, 2013). Further investigation with samples from relapsed ALL is needed to elucidate the effect of these clones and subclones on disease progression and whether they can provide clues to the cause of treatment failure (Meyer et al., 2013; Tzoeva et al., 2013; Lindqvist et al., 2015). Unfortunately these samples were not available in this study.

The present study showed that TP53 mutations are the mutations most frequently observed in B-ALL patients. The tumour suppressor gene TP53 has a crucial role in cell cycle regulation and apoptosis after DNA damage, and its role in tumorigenesis is well recognised in solid and haematological malignancies (Chiaretti et al., 2013). In particular, TP53mut was associated with poor outcome in the whole cohort of patients and when considering adults and children separately. These results are in agreement with those of previous studies in which TP53mut was associated with resistance to treatment and worse prognosis in several tumours (Olivier et al., 2010; Salmoiraghi et al., 2016). In addition, alterations of the TP53 gene were described as being important at relapse in childhood and adult ALL, in which they independently predict a high risk of treatment failure in a significant number of patients (Hof et al., 2011). The presence of TP53 mutations was also associated with a reduced response rate to induction therapy (Chiaretti et al., 2013) and a shorter survival (from time of diagnosis and from time of relapse), even after successful reinduction therapy (Dicianni et al., 1994). These lines of evidence, together with our results, highlight the importance of sequencing TP53 at diagnosis.

| Parameter | Univariate analysis P | HR | CI (95%) | P   |
|-----------|----------------------|----|----------|-----|
| TP53mut  | 0.019                | 2.3 | 1.1–5.1  | 0.035 |
| Poor risk cytogenetic | 0.013 | 1.7 | 1.1–2.9  | 0.02  |
| WBC ≥ 30 × 10^9l^-1 | 0.288 |     |          |      |
| MRD ≥ 0.1% | 0.314 |     |          |      |
| Pro-B B-ALL | 0.923 |     |          |      |
| JAK2mut | 0.972                |    |          |      |
| CRLF2mut | 0.641                |    |          |      |

**Table 3. Univariate and multivariate survival analysis in adults with B-ALL**

**Multivariate analysis with OS**

| Parameter | Univariate analysis P | HR | CI (95%) | P |
|-----------|----------------------|----|----------|---|
| TP53mut  | 0.025                | 2.3 | 1.1–4.9  | 0.028 |
| WBC ≥ 30 × 10^9l^-1 | 0.008 | 1.2 | 0.6–2.5  | 0.66  |
| MRD ≥ 0.1% | 0.023 | 2.1 | 1.0–4.0  | 0.036 |
| Pro-B B-ALL | 0.516 |     |          |      |
| TP53mut  | 0.019                | 2.3 | 1.1–5.1  | 0.035 |
| Poor risk cytogenetic | 0.013 | 1.7 | 1.1–2.9  | 0.02  |
| WBC ≥ 30 × 10^9l^-1 | 0.288 |     |          |      |
| MRD ≥ 0.1% | 0.314 |     |          |      |
| Pro-B B-ALL | 0.923 |     |          |      |
| JAK2mut | 0.972                |    |          |      |
| CRLF2mut | 0.641                |    |          |      |

**Multivariate analysis with EFS**

| Parameter | Univariate analysis P | HR | CI (95%) | P |
|-----------|----------------------|----|----------|---|
| TP53mut  | 0.029                | 5.9 | 1.2–28.6 | 0.027 |
| JAK2mut  | 0.002                | 5.6 | 1.1–28.1 | 0.036 |
| WBC ≥ 30 × 10^9l^-1 | 0.024 | 2.2 | 0.9–5.1  | 0.072 |
| MRD ≥ 0.1% | 0.003 | 2.4 | 1.1–5.3  | 0.026 |
| Pro-B B-ALL | 0.599 |     |          |      |
| Poor risk cytogenetic | 0.988 |     |          |      |
| CRLF2mut | 0.412                |    |          |      |

**Multivariate analysis with RR**

Abbreviations: B-ALL = B-cell precursor acute lymphoblastic leukaemia; CI = confidence interval; EFS = event-free survival; HR = hazards ratio; MRD = minimal residual disease; OS = overall survival; RR = relapse rate; WBC = white blood cell. The parameters with P-values < 0.05 were considered as statistically significant in the univariate analysis and were included in multivariate analysis. Significant parameters are highlighted in bold.
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Recurrent fusion genes. Given the negative influence on outcome, we reviewed reports (Mullighan et al., 2007; Vainchenker and Constantinescu, 2013). The acquired JAK2mut (p.R683S and p.R683G) is presumed to be a biomarker for B-ALL (Bercovich et al., 2008; Mullighan, 2008; Mullighan et al., 2009b). Recent studies suggested that the amino acid residue p.R683 located in the linker between the N and C lobes of JH2 domain is important for maintaining the activity, structural stability and folding of JAK2 (Li et al., 2013). The mutations in p.R683 disrupted the structure of JH2 domain leading to JAK2 constitutive activation and induced growth factor-independent cell proliferation of the mouse Ba/F3 hematopoietic cell line (Mullighan, 2008; Li et al., 2013). In our study, two B-ALL patients with two independent clones each, displayed different mutations in the p.R683 residue (Bercovich et al., 2008). Even though the oncogenic effect of p.R683S and p.R683G mutations could be similar or identical, further research will be necessary to fully understand the biological relevance of this clonal heterogeneity. The development of JAK2 inhibitors that abrogates JAK/STAT activation may be a useful approach for treating patients harbouring these mutations (Mullighan et al., 2009b; Roberts and Mullighan, 2011).

We found that CRLF2oe is associated with JAK2 mutations in B-ALL in cases lacking recurrent gene fusions, according to previous reports (Mullighan et al., 2009a; Yoda et al., 2010; Harvey et al., 2010). In our study, the frequency of CRLF2oe found in children and adults was around 17%. While in children it was similar to that observed by Chen et al. (2012), in adults was lower (Chiaretti et al., 2016) even though maintained its association with poor outcome in this group of patients. Therefore, CRLF2 quantification could be an important prognostic marker in adult B-ALL. Evidence from in vitro studies suggests that these proteins could cooperate to transform B-ALL cells as CRLF2 acts as a scaffold for JAK2mut signalling (Yoda et al., 2010), although the precise molecular mechanism remains undescribed. However, cells dependent on CRLF2 signalling are also sensitive to JAK2 inhibitors. Thus, ALL patients with CRLF2oe may benefit from future kinase inhibitor approaches (Roll and Reuther, 2010; Yoda et al., 2010). Future studies should be conducted to further elucidate JAK2/CRLF2 association and prognosis in B-ALL. In this sense, further assessment of gene rearrangements involving CRLF2 as IGH@-CRLF2 and P2RY8-CRLF2 should be performed to establish the prognostic significance of CRLF2oe and CRLF2 rearrangements across risk subgroups of B-ALL.

CONCLUSIONS

The amplicon NGS results indicate that the incidence of mutation in TP53, JAK2, IL7R, PAX5, LEFI and CRLF2 is higher in adults than in children. All mutations were frequent in B-ALL cases without recurrent fusion genes. Given the negative influence on outcome, we suggest that TP53 and JAK2 status should be investigated at diagnosis, particularly in patients negative for recurrent fusion genes, for whom genetic-based prognostic stratification is still limited. Deep sequencing may ultimately guide treatment decisions better for B-ALL patients bearing TP53 and JAK2 mutations and could give rise to alternative therapeutic regimens.

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DECLARATIONS

Ethics approval and consent to participate: The study was approved by the local ethical committee, the Comite Tecnico de Investigacion Clinica del area de salud de Salamanca, at the Hospital Universitario de Salamanca. Written informed consent was obtained from each patient or legal guardians before patients entered the study. Availability of data and material: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MFC wrote the paper and edited the manuscript. MFC, IBM, SR and EFR recorded and analysed the clinical and biological data. MFC, CR, RB, MA, MHS, JMHS, MQA, IBM, MSV and IR designed, performed and analysed the NGS experiments. IBM, MSV and FAS performed the CRLF2 expression studies. JLF, MArf, NH, JNR, LH, JR, IR, JMSP, MC, MR, CD, EB, JM, JMR and JMHR provided patient samples and the clinical data. AK provided reagents and supported NGS analysis; all authors participated in discussions and critically reviewed the manuscript; JM, EFR, AK and JMHR analysed and interpreted the data, led and supervised the study and corrected and approved the final version of the manuscript.

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