CD200/BTLA deletions in pediatric precursor B-cell acute lymphoblastic leukemia treated according to the EORTC-CLG 58951 protocol

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ABSTRACT

DNA copy number analysis has been instrumental for the identification of genetic alterations in B-cell precursor acute lymphoblastic leukemia. Notably, some of these genetic defects have been associated with poor treatment outcome and might be relevant for future risk stratification. In this study, we characterized recurrent deletions of *CD200* and *BTLA* genes, mediated by recombination-activating genes, and used breakpoint-specific polymerase chain reaction assay to screen a cohort of 1154 cases of B-cell precursor acute lymphoblastic leukemia uniformly treated according to the EORTC-CLG 58951 protocol. *CD200/BTLA* deletions were identified in 56 of the patients (4.8%) and were associated with an inferior 8-year event free survival in this treatment protocol [70.2% ± 1.2% for patients with deletions *versus* 83.5% ± 6.4% for non-deleted cases (hazard ratio 2.02; 95% confidence interval 1.23-3.32; P=0.005)]. Genetically, *CD200/BTLA* deletions were strongly associated with *ETV6-RUNX1*-positive leukemias (P<0.0001), but were also identified in patients who did not have any genetic abnormality that is currently used for risk stratification. Within the latter population of patients, the presence of *CD200/BTLA* deletions was associated with inferior event-free survival and overall survival. Moreover, the multivariate Cox model indicated that these deletions had independent prognostic impact on event-free survival when adjusting for conventional risk criteria. All together, these findings further underscore the rationale for copy number profiling as an important tool for risk stratification in human B-cell precursor acute lymphoblastic leukemia. *This trial was registered at www.ClinicalTrials.gov as #NCT00003728.*

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer of childhood.[1] The prognosis of this disease has improved greatly over the last 40 years and cure rates currently exceed 80%.[2] The 8-year event-free survival and overall survival rates in treatment protocol 58951 of the Children's Leukemia Group (CLG) of the European Organization for Research and Treatment of Cancer (EORTC) reached 81% and 89%, respectively.[3-5] Improvements in diagnosis, novel insights into disease biology and, most importantly, better risk stratification, have contributed enormously to this success.[5,6]

In current treatment protocols, high-risk ALL is defined by hypodiploidy/near-hypodiploidy, presence of the chromosomal translocation t(9;22)(*BCR–ABL1*), mixed-lineage leukemia (MLL) gene rearrangements, poor pre-phase response, absence of complete remission after induction treatment or high levels of minimal residual disease. Although the relative percentage of hematologic relapses in ALL is clearly greater in high-risk patients, the absolute number of disease recurrences is actually higher in non-high-risk patients, who account for up to 75% of cases of pediatric ALL and who predominantly have ALL of B-cell lineage.[7] Identification of additional prognostic markers that could be used for risk stratification in non-high-risk B-cell precursor (BCP)-ALL is, therefore, of utmost importance.

Recent genome-wide screening approaches have identified

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several recurring genetic alterations in BCP-ALL, some of which show prognostic relevance.[8] These cooperative somatic lesions include copy number alterations and mutations targeting tumor suppressors, cell-cycle and apoptotic regulators, drug-receptor genes and lymphoid-signaling molecules.[9,10] For example, more than 40% of BCP-ALL patients harbor mutations in critical transcriptional regulators of B lymphoid development including Paired Box 5 (*PAX5*), Early B-Cell Factor 1 (*EBF1*) and IKAROS Family Zinc Finger 1 (*IKZF1*). Notably, *IKZF1* deletions have convincingly been associated with poor prognosis in both *BCR–ABL1*-positive as well as unselected BCP-ALL cohorts of patients.[11,12] Of note, two independent studies recently showed that intragenic *ERG* deletions attenuate the unfavorable impact of *IKZF1* deletions, suggesting that full genomic profiling is important in studying prognostic factors in BCP-ALL.[3,13]

In this study, we performed high-resolution copy number analysis on leukemic specimens from 70 average-risk pediatric BCP-ALL patients treated according to the EORTC-CLG 58951 protocol. In this initial cohort, previously reported genomic deletions encompassing the B and T lymphocyte attenuator (*BTLA*; also called *CD272*) and *CD200* genes[2,14-18] were associated with a high incidence of hematologic relapse. Subsequently, we extended the cohort to 1154 BCP-ALL patients treated on the EORTC-CLG 58951 protocol to evaluate the biological and clinical relevance of *CD200/BTLA* deletions in BCP-ALL.

Methods

Full details of the methods are provided in the *Online Supplementary Methods*.

Patients

Patients under 18 years of age with previously untreated ALL were enrolled in the EORTC-CLG 58951 trial between December 1998 and July 2008. This protocol was approved by the EORTC Protocol Review Committee and by the local institutional ethical committees in each participating center and is registered at ClinicalTrials.gov (#NCT00003728). After obtaining informed consent from patients or their legal guardians according to the declaration of Helsinki, bone marrow and blood samples were taken at diagnosis, before the start of treatment, and stored for future research purposes. All analyses were performed on cryopreserved leukemic cells.

Diagnostics, risk group assignment and treatment were performed according to the EORTC-CLG 58951 protocol. This protocol is based on a Berlin-Frankfurt-Munster (BFM)-backbone with four-drug induction, consolidation, central nervous system prophylaxis without cranial irradiation, re-induction/late intensification and maintenance, and includes three randomized comparisons as described previously.[5,19]

Array comparative genome hybridization

The DNA of samples and cell lines was profiled on the 180K custom-designed oligonucleotide array platform. In brief, genomic DNA from patients and controls was labeled using random prime labeling with Cy3 and Cy5 dyes (Perkin Elmer, Waltham, MA, USA). Next, hybridization was performed according to the manufacturer's instructions (Agilent Technologies) and then the data were analyzed using the arrayCGHbase tool, developed in-house.[20] In addition, a custom focused 8*15K oligonucleotide array (Agilent Technologies, Santa Clara, CA, USA) was designed for fine-mapping the deletion encompassing *BTLA* and *CD200*.

High throughput screening for CD200/BTLA deletions

Primers flanking the junctions were designed based on breakpoint sequences, using Primer3Plus software and included: F1: 5'-TTTCCGGAGTCTCAGAGAGGT -3', F2: 5'-AGGCTTTGCTTCGTCTTCTG-3', and R(6-FAM-labeled): 5'-TTAAAGACGGCTGCTCTTCC -3'.

Fifty nanograms of DNA were amplified using standard polymerase chain reaction (PCR) procedures, with 1.5 mM $MgCl_2$ and an annealing temperature of 60°C. Fluorescent PCR products were analyzed using the ABI 3130 analyzer and GeneMapper® software (Applied Biosystems).

Statistical analysis

Event-free survival was calculated from the date of diagnosis to the date of last follow up or the first event (no complete remission, relapse or death). Disease-free survival was calculated from the date of complete remission to the date of last follow-up or the first event (relapse or death). All patients alive and in first complete remission were censored at their last follow-up. Overall survival was calculated from the date of diagnosis until the date of death. Patients still alive were censored at their last follow-up. Survival rates were calculated using the Kaplan-Meier technique, and standard errors of estimates were obtained from the Greenwood formula. Differences between Kaplan-Meier curves were tested for statistical significance using the two-tailed log-rank test. The Cox proportional hazard model was used to obtain the estimate and the 95% confidence interval (CI) of the hazard ratio (HR) of the instantaneous event rate in one group *versus* another, as specified by a given variable. All analyses were based on the intent-to-treat principle. The relationship between presence/absence of *CD200/BTLA* deletions and categorical variables was tested for significance using the Fisher test, and for continuous variables (e.g. age) using the Wilcoxon test.

Results

Recurrent CD200/BTLA deletions in average-risk precursor-B cell acute lymphoblastic leukemia

Copy number profiling was performed on leukemic blasts from 70 children with average-risk BCP-ALL treated with the EORTC-CLG 58951 protocol. Array comparative genome hybridization (CGH) analysis revealed a recurrent deletion of a genomic region (~164kb) on the long arm of chromosome 3 (3q13.2) in seven out of 70 (10%) patients; the deletion encompassed the lymphoid signaling molecules *BTLA* (*CD272*) and *CD200* genes. In this series, five out of seven deletions were mono-allelic for both genes (Figure 1A), whereas the two other samples showed mono-allelic loss of *CD200* in combination with bi-allelic deletion of *BTLA* (Figure 1B). Furthermore, copy number profiling in human BCP-ALL cell lines revealed similar bi-allelic *CD200/BTLA* deletions in the *ETV6-RUNX1*-positive REH cell line (Figure 1C).

To evaluate somatic mutations as an alternative mechanism of *BTLA* or *CD200* inactivation in pediatric BCP-ALL, we performed sequencing of all coding exons of *BTLA* and *CD200* in the same panel of 70 average-risk pediatric BCP-ALL patients (*Online Supplementary Table S1*). This analysis failed to identify somatic loss-of-function mutations targeting *BTLA* or *CD200* (*Online Supplementary Table S2*).

Next, we evaluated the functional consequences of *CD200/BTLA* deletions on the expression levels of both lymphoid signaling factors. First, quantitative reverse transcriptase PCR in a panel of BCP-ALL cell lines showed that *CD200/BTLA* deletions completely abolished *CD200* and *BTLA* expression in the REH cell line, whereas their expression levels were variable in the non-deleted cell lines (Figure 1D). In addition, these results were confirmed at the protein level by flow cytometry. Notably, CD200 expression was usually strong, whereas BTLA was virtually absent across the panel of BCP-ALL cell lines (Figure 1E). Finally, similar analyses were performed on leukemic lymphoblasts from 23 patients with primary BCP-ALL, including three who harbored mono-allelic *CD200/BTLA* deletions. Analysis of $CD19^{+}$ $CD3^{-}$ leukemic lymphoblasts revealed low levels of BTLA in all BCP-ALL samples without significant differences in BTLA expression between deleted (mean fluorescence intensity, MFI: 198±109) and non-deleted leukemias (MFI: 378±74). In contrast, CD200 protein expression was more prominent in all primary BCP-ALL blasts with a clear decrease in MFI in deleted BCP-ALL cases (MFI in deleted cases: 2830±1110 *versus* MFI in non-deleted cases: 6730±980) (Figure 1F) (*Online Supplementary Table S3*).

Genomic breakpoints of CD200/BTLA deletions in precursor-B cell acute lymphoblastic leukemia

To map the exact genomic breakpoints of *CD200/BTLA* deletions in BCP-ALL, we developed a custom array with high-density probe coverage at chromosomal band 3q13.2 surrounding the *CD200* and *BTLA* genes. Notably, exact recombination-activating genes (RAG) recognition sequences were identified at both proximal and distal breakpoints with some random nucleotides inserted at the junctions, suggesting that aberrant activity of RAG enzymes might act as the driving mechanism for

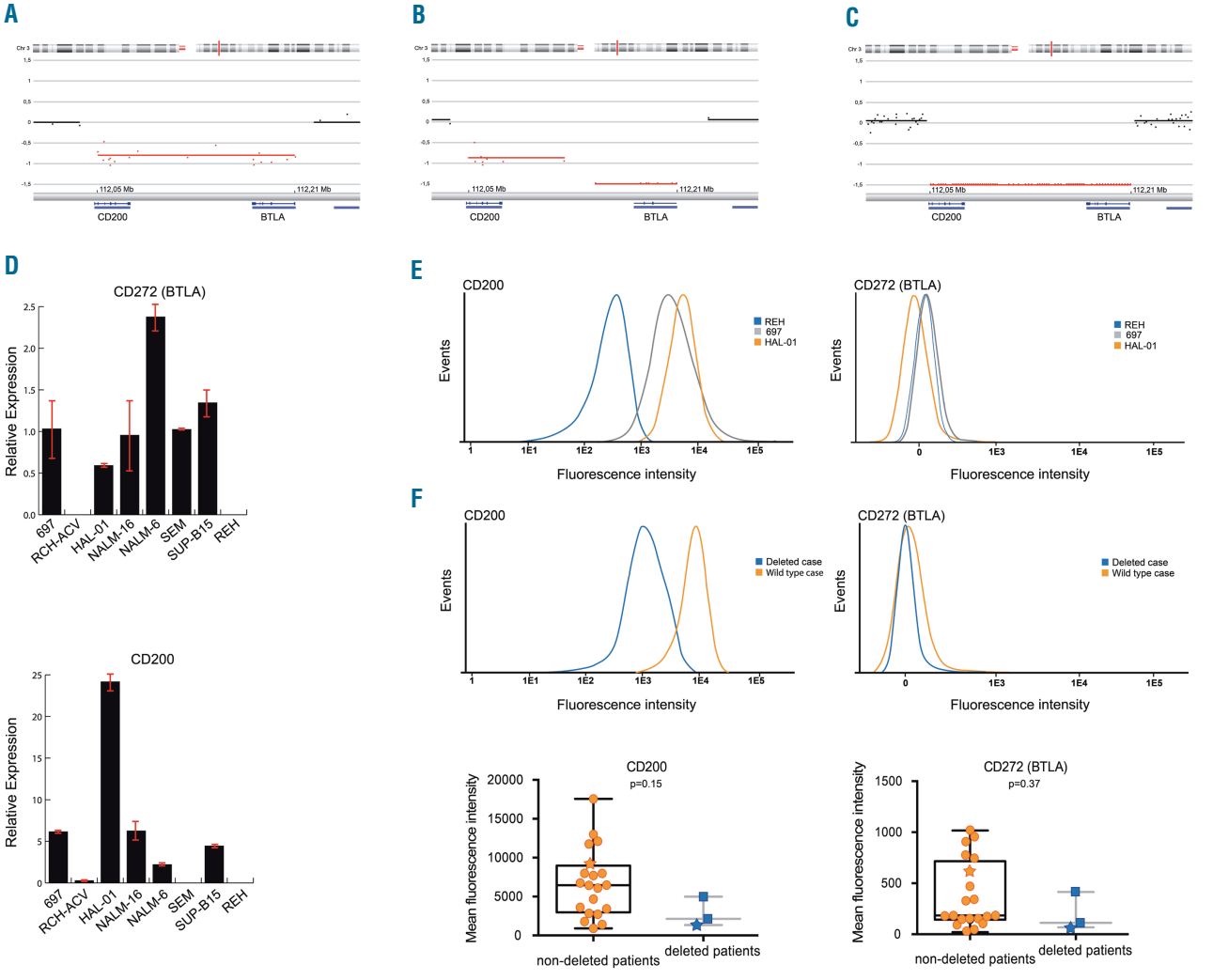

Figure 1. Characterization of focal deletions of *CD200/BTLA*. CGH log_2 copy number ratio data shown as representation for (A) a mono-allelic deletion of *CD200* and *CD272 (BTLA)* genes, (B) a mono-allelic deletion of *CD200* gene and a bi-allelic deletion of *CD272 (BTLA)* in two different BCP-ALL cases and (C) a bi-allelic deletion of *CD200* and *CD272 (BTLA)* in the REH cell line. (D) Quantitative reverse transcriptase-PCR analysis in eight B-ALL cell lines confirmed that deletion of *CD200* and *CD272 (BTLA)* abolishes their expression in the REH cell line. The expression profiles of *CD200* and *CD272 (BTLA)* are shown on a logarithmic scale in (E) three B-ALL cell lines and (F) BCP-ALL patients. The top panel shows the expression level of *CD200* and *CD272 (BTLA)* in one wild-type and one deleted case and the box plots in the lower panel represent the mean fluorescence intensity of *CD200* and *CD272 (BTLA)* in three deleted and 20 non-deleted BCP-ALL cases. Asterisks indicate samples used to generate the top panels.

CD200/BTLA deletions in BCP-ALL (Figure 2A).

Next, we developed primers near the breakpoint cluster regions as a strategy to identify the exact genomic breakpoint sequences of *CD200/BTLA* deletions by PCR analysis and Sanger sequencing (Figure 2B). Analysis of five cases of *CD200/BTLA* deletion-positive BCP-ALL and the REH cell line showed that the genomic breakpoints of the *CD200/BTLA* deletions were conserved with minimal differences between *CD200/BTLA* deletions from different BCP-ALL patients (Figure 2C).

Clinical and biological features of CD200/BTLA-deleted precursor-B cell acute lymphoblastic leukemia

In the initial cohort, *CD200/BTLA* deletions were associated with a high incidence of hematologic relapse (4/7), whereas only eight out of 63 patients without a *CD200/BTLA* deletion relapsed. To evaluate the clinical and biological features of *CD200/BTLA* deletion-positive BCP-ALL, we extended the cohort to 1154 BCP-ALL patients treated according to the EORTC-CLG 58951 protocol and screened for the presence of *CD200/BTLA* deletions. To this end, we optimized a breakpoint-specific fluorescent PCR assay based on the primer combinations used for the genomic breakpoint characterization (Figure 2B).

CD200/BTLA deletions were detected in 56 out of 1154 patients (4.8%); the patients' clinical and biological features according to *CD200/BTLA* status are presented in Table 1. No significant differences were observed between patients with and without *CD200/BTLA* deletions. However, whereas pre-phase response was similar in patients with and without deletions, a higher proportion of patients with minimal residual disease levels ≥10^{-2} after

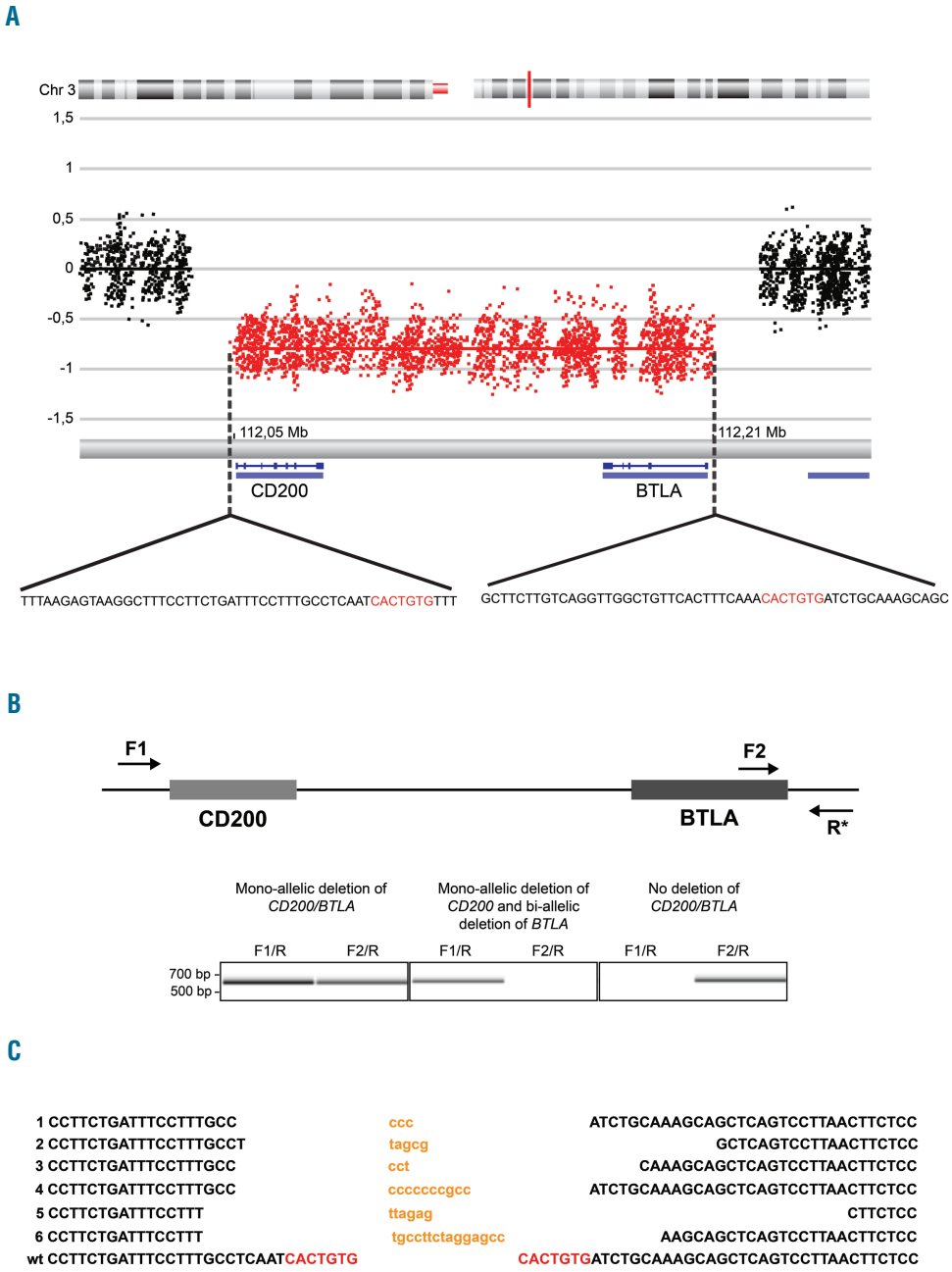

Figure 2. Genomic breakpoints of *CD200/BTLA* deletions in BCP-ALL patients. (A) Custom oligo array with high-density probe coverage at ch3q13.2, the RAG recognition sequences were identified within the deleted region. Each small vertical blue line indicates an exon. The thicker the line, the larger the exon. (B) Schematic representation of the *CD200* and *BTLA* genes with locations of primers used for breakpoint-specific multiplex fluorescent PCR assay. Boxes represent genes, the arrows represent the primers and the star represents the fluorescent-labeled primer. Patients with mono-allelic deletion of both genes show both bands of F1/R and F2/R, patients with mono-allelic deletion of *CD200* and bi-allelic deletion of *BTLA* show only the band of F1/R, patients with no deletion show only the band of F2/R. (C) Alignment of breakpoint sequences of five cases of *CD200/BTLA* deletion-positive BCP-ALL (n.1-5) and the REH cell line (n.6) with wild-type sequence showed that the genomic breakpoints of the *CD200/BTLA* deletions were conserved.

induction was found in the *CD200/BTLA*-deleted group (7.1% *versus* 2.65%; *P*=0.54, Table 1). Diagnostic samples were not available from 98 BCP-ALL patients in the 58951 protocol. The characteristics of these children did not differ from those of the tested cohort (*Online Supplementary Table S4*).

The distribution of *CD200/BTLA* non-deleted *versus* deleted cases among well-known genetic aberrations and different genetic risk groups is shown in Table 2. The definition of genetic risk groups is based on internationally accepted prognostic associations of the main classifying genetic lesions. The good-prognosis genetic group includes patients with high hyperdiploidy, *ETV6–RUNX1* translocation or *ERG* deletion,[3] whereas the poor-prognosis genetic group consists of patients with *BCR-ABL* translocation, near-haploidy, low hypodiploidy, *MLL* translocations or iAMP21. The remaining patients (*TCF3-PBX1* and B-other) are classified in the intermediate-prognosis genetic group.

The highest frequency of *CD200/BTLA* deletions was seen in the poor-prognosis genetic group (8/79=10.1%). The deletions were detected in 5.5% (39/705) and 2.4% (9/370) of the good-prognosis and intermediate-prognosis genetic groups, respectively. The highest number of *CD200/BTLA*-deleted cases (39/56, 69.6%) was found in the good-prognosis genetic group with a striking association of *CD200/BTLA* deletions with the presence of the *ETV6/RUNX1* fusion gene (34/282, *P*<0.0001). Significantly fewer cases of *CD200/BTLA* deletions were seen among high-hyperdiploid patients (4/386, *P*=0.0001) (Table 2).

Table 1. Clinical and biological features of BCP-ALL patients according to *CD200/BTLA* deletions status.

Characteristic	Not deleted (n=1098) N. (%)	Deleted (n=56) N. (%)	*P* value
Gender			
Male	587 (53.5)	30 (53.6)	1
Female	511 (46.5)	26 (46.4)	
Age at diagnosis (years):			0.8
<1	3 (0.3)	0 (0)	
1 – < 10	880 (80.2)	44 (78.6)	
≥10	215 (19.6)	12 (21.4)	
WBC count (x10⁹/L):			0.5
<10	617 (56.2)	28 (50.0)	
10-<25	214 (19.5)	15 (26.8)	
25-<100	209 (19.0)	8 (14.3)	
≥100	58 (5.3)	5 (8.9)	
NCI risk group:			0.55
Standard risk	772 (70.3)	37 (66.1)	
High risk	326 (29.7)	19 (33.9)	
Pre-phase response:			0.88
<1x10⁹/L	1027 (93.5)	53 (94.6)	
≥1x10⁹/L	71 (6.5)	3 (5.4)	
MRD after induction:			0.54
≥10^{-2}	29 (2.6)	4 (7.1)	
10^{-2} to 10^{-3}	64 (5.8)	4 (7.1)	
Undetectable	877 (79.9)	41 (73.2)	
Missing	128 (11.7)	7 (12.5)	
Initial CNS score			0.67
CNS-1	1028 (93.6)	54 (96.4)	
CNS-2/TLP+	50 (4.6)	1 (1.8)	
CNS-3	16 (1.5)	0 (0.0)	
Missing	4 (0.4)	1 (1.8)	

WBC: white blood cell; NCI: National Cancer Institute; MRD: minimal residual disease; CNS: central nervous system; TLP: traumatic lumbar puncture.

Prognostic relevance of CD200/BTLA deletions in precursor-B cell acute lymphoblastic leukemia

Survival analyses were performed to evaluate the prognostic relevance of *CD200/BTLA* deletions in the whole BCP-ALL cohort. Figure 3 shows the event-free survival, disease-free survival and overall survival curves of the patients according to *CD200/BTLA* status, with a median follow-up of 6.9 years. The 8-year event-free survival rate was 83.5% (95% CI 80.9-85.7) for patients without *CD200/BTLA* deletions whereas it was only 70.2% (95% CI 55.8-80.8) in the group with *CD200/BTLA* deletions (HR 2.02; 95% CI 1.23-3.32; *P*=0.005). There were 17 events in the group of 56 patients with *CD200/BTLA* deletions whereas 168 events were seen in the 1098 patients without *CD200/BTLA* deletions. Survival analysis from complete remission showed that the *CD200/BTLA* deletions were significantly associated with inferior 8-year disease-free survival within the whole group of patients (72.8% *versus* 84.4%; HR 1.92; 95% CI 1.13-3.26; *P*=0.014). In contrast, no significant difference was observed in the 8-year overall survival rate between BCP-ALL patients with and without *CD200/BTLA* deletions (91.4% *versus* 87%; HR 1.57; 95% CI 0.73-3.4; *P*=0.247). Event status and relapse type for the groups of patients with and without deletions are summarized in Table 3.

Eight-year event-free survival and overall survival rates were significantly different in previously defined good-prognosis, intermediate-prognosis and poor-prognosis genetic groups (*Online Supplementary Figure S1A*). The occurrence of *CD200/BTLA* deletions did not influence event-free survival rates of the patients in the good-prognosis genetic group (HR 1.38; 99% CI 0.41-4.57; *P*=0.49) nor did it influence overall survival rate (HR 0.65; 99% CI 0.05-8.96; *P*=0.66) (*Online Supplementary Figure S1B*). In the poor-prognosis genetic group, patients with *CD200/BTLA*

Table 2. Frequency of *CD200/BTLA* deletions according to BCP-ALL genetic subtypes and genetic risk groups.

	Not deleted (n=1098) N. (%)	Deleted (n=56) N. (%)	*P* value
Genetic subtypes			<0.0001
ERG^{del}	36 (3.3)	1 (1.8)	
ETV6-RUNX1	248 (22.6)	34 (60.7)	
High hyperdiploidy	382 (34.8)	4 (7.1)	
BCR-ABL1	24 (2.2)	3 (5.4)	
Low hypo/near-haploidy	9 (0.8)	1 (1.8)	
MLL translocation	17 (1.5)	0 (0.0)	
iAMP21	21 (1.9)	4 (7.1)	
TCF3-PBX1	47 (4.3)	0 (0.0)	
B-other	314 (28.6)	9 (16.1)	
IKZF1^{del}	172 (15.7)	10 (17.9)	0.7
Genetic groups *			0.004
Good-prognosis	666 (60.7)	39 (69.6)	
Intermediate-prognosis	361 (32.9)	9 (16.1)	
Poor-prognosis	71 (6.5)	8 (14.3)	

Genetic risk groups were defined as follows: good-prognosis group includes all patients with high hyperdiploidy, ETV6-RUNX1 *or* ERG^{del}*; intermediate-prognosis group includes patients with* TCF3-PBX1 *and B-other patients; poor-prognosis group includes all patients with* MLL *translocation,* BCR-ABL, *low hypodiploidy/near-haploidy or iAMP21.*

deletions tended towards a worse 8-year event-free survival rate (HR 1.80; 99% CI 0.57-5.70; *P*=0.18) while overall survival was barely influenced (HR 1.02; 99% CI 0.15-7.03; *P*=0.97) (*Online Supplementary Figure S1C*). Notably, in the intermediate-prognosis genetic group, *CD200/BTLA* deletions were significantly associated with an inferior 8-year event-free survival [33.3% (95% CI 7.8-62.3) *versus* 76.2% (95% CI 71.0-80.6); HR 4.00 (99% CI 1.34-11.93); *P*<0.001] and an inferior 8-year overall survival [48.6% (95% CI 12.8-77.6) *versus* 86.9% (95%CI 82.6-90.2); HR 4.43 (99%CI 1.15-17.03); *P*=0.002] (Figure 4A,B). No significant differences in leukemic and clinical characteristics were observed between patients with and without *CD200/BTLA* deletions in the intermediate-prognosis genetic group (*Online Supplementary Table S5*). In the intermediate-prognosis genetic group, comparing the patients with and without *CD200/BLTA* deletions, the former had higher incidences of not achieving complete remission (2/9 *versus* 7/361) and of relapsing (4/9 *versus* 67/361). Both children with *CD200/BTLA* deletions who failed to achieve complete remission died during induction, one from sepsis and the other from necrotising enterocolitis. A similar failure to achieve complete remission was observed in three patients in the non-deleted group. Within the intermediate-prognosis genetic group the disease-free survival rates of patients with and without *CD200/BTLA* deletions were 42.9% *versus* 77.7%, respectively (HR 3.03; 99% CI 0.81-11.41; *P*=0.02).

Finally, multivariate analysis was performed to evaluate the prognostic role of *CD200/BTLA* deletions after adjusting for conventional risk criteria (Table 4). In the whole cohort, *CD200/BTLA* deletions were independently associated with inferior 8-year event-free survival (HR 1.97; 95% CI 1.17-3.32; *P*=0.01) and disease-free survival (HR 1.83; 95% CI 1.05-3.18; *P*=0.03) as shown in a Cox regression model including gender, *IKZF1* status, response to pre-phase and genetic risk groups.

The inferior event-free survival was confirmed for the *CD200/BTLA*-deleted patients in the intermediate-prognosis genetic group (HR 2.99; 95% CI 0.97-9.31; *P*=0.01), whereas disease-free survival was comparable with that of the patients without *CD200/BTLA* deletions (HR 2.11; 99% CI 0.54-8.29; *P*=0.16).

Table 3. An overview of event status and relapse types in BCP-ALL patients.

Characteristic	Not deleted n=1098 N. (%)	Deleted n=56 N. (%)
No CR+ induction death	12 (1.1)	2 (3.6)
Relapse:	143 (13.0)	14 (25.0)
BM	96 (8.7)	10 (17.9)
CNS	10 (0.9)	1 (1.8)
Other isolated	8 (0.7)	0 (0)
BM+CNS	15 (1.4)	3 (5.4)
BM+other	13 (1.2)	0 (0)
Unknown	1 (0.1)	0 (0)
Treatment-related mortality	13 (1.2)	1 (1.8)
Continued complete remission	930 (84.7)	39 (69.9)
Survival status:		
Alive	1012 (92.2)	49 (87.5)
Dead	86 (7.8)	7 (12.5)

CR: complete remission; BM: bone marrow; CNS: central nervous system.

Discussion

Genomic profiling studies have uncovered a plethora of genomic lesions involved in the pathogenesis of BCP-ALL.[21] Notably, some of these genetic alterations are clinically relevant and might be included in future stratification strategies for the treatment of pediatric BCP-ALL. Of particular interest, *IKZF1* alterations have been found in *BCR–ABL*-positive[12,22] and in a small fraction of *BCR–ABL*-negative[23] BCP-ALL and have shown predictive value for

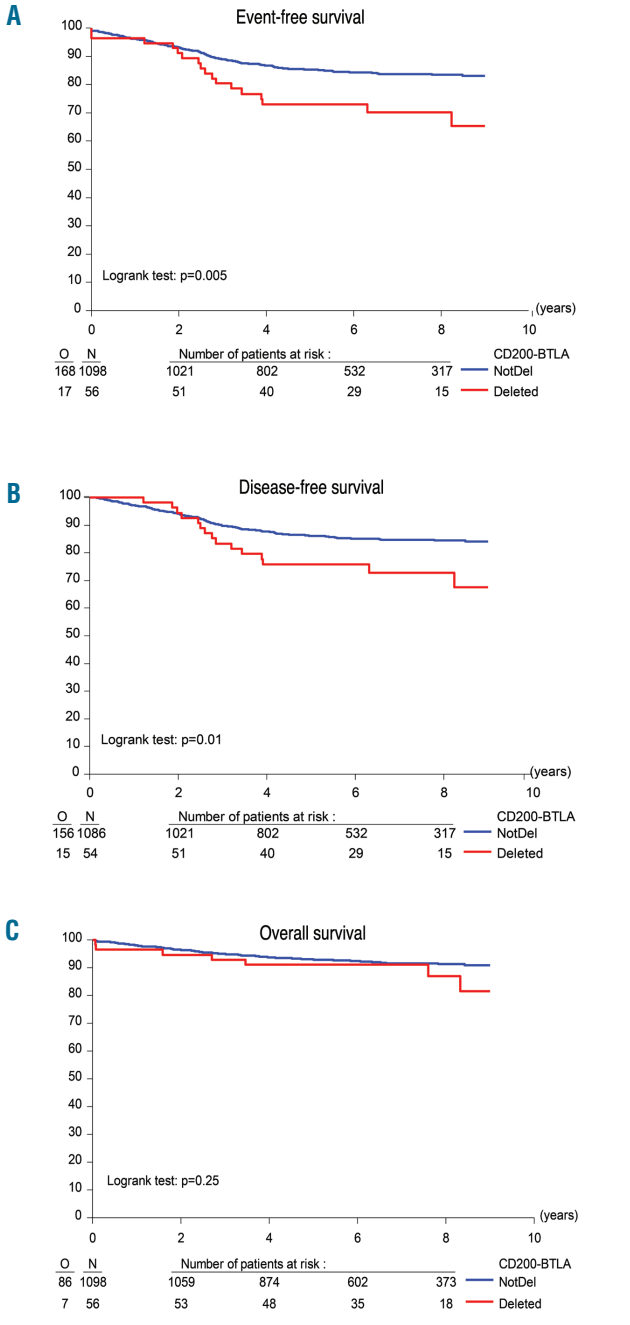

Figure 3. Associations between *CD200/BTLA* deletions and outcome of all BCP-ALL patients treated according to the EORTC 58951 trial. Kaplan-Meier curves of (A) event-free survival, (B) disease-free survival and (C) overall survival in all BCP-ALL patients according to the presence of *CD200/BTLA* deletions.

poor treatment outcome in several studies.[2,11,16,24,25] In contrast, *ERG* deletions were shown to be associated with good treatment response.[3,13] In addition, Clappier and colleagues showed that the presence of *IKZF1* deletions failed to influence the good treatment outcome generally observed in *ERG*-deleted BCP-ALL patients.[3] All together, these results suggest that in-depth genomic profiling of primary BCP-ALL is clinically important and can provide new opportunities for further improvement of BCP-ALL risk stratification.

In this study, we characterized a recurrent somatic deletion of approximately 164 kb at the 3q13.2 locus. This deletion targets *BTLA* and *CD200* and occurred in 10% of our initial cohort (7 out of 70) and in 4.85% of our extended validation cohort (56 out of 1154). *CD200/BTLA* deletions have been previously reported in independent genome-wide copy number profiling studies in BCP-ALL.[2,14-18] Importantly, both *BTLA* and *CD200* encode lymphoid signaling molecules with putative functions in B-cell receptor and MAPK/PI3K signaling, both of which are essential for B-cell proliferation and maturation.[26,27] A high frequency of focal *CD200/BTLA* deletions in combination with the lack of somatic mutations targeting these genes, suggests that combined loss of both lymphoid signaling molecules might be required during leukemogenesis. Alternatively, the deletion of both genes might be required because of the presence of a RAG signal sequence and thus might represent an epiphenomenon of another event in the process of leukemogenesis. *In vivo* experiments will enable the assessment of the validity of these hypotheses.

CD200/BTLA deletions clearly affected CD200 expression levels in B-ALL cell lines and in patients with primary BCP-ALL. Moreover and in line with the variable pattern of CD200 expression that has been reported in B and T cells,[28] BCP-ALL patients and B-ALL cell lines that lacked *CD200/BTLA* deletions were also characterized by a variable range of CD200 expression levels. Notably, low or absent CD200 expression in these *CD200/BTLA*-deletion-negative BCP-ALL patients and cell lines suggests that alternative mechanisms, such as epigenetic silencing, might be implicated in CD200 inactivation.[29] In contrast, the levels of BTLA (CD272) were generally low in all cell lines and BCP-ALL patients' samples analyzed, which corresponds to the marginal levels of BTLA detected in pro, pre and immature B cells.[26] All together, these expression data seem to suggest that BTLA only plays a marginal role

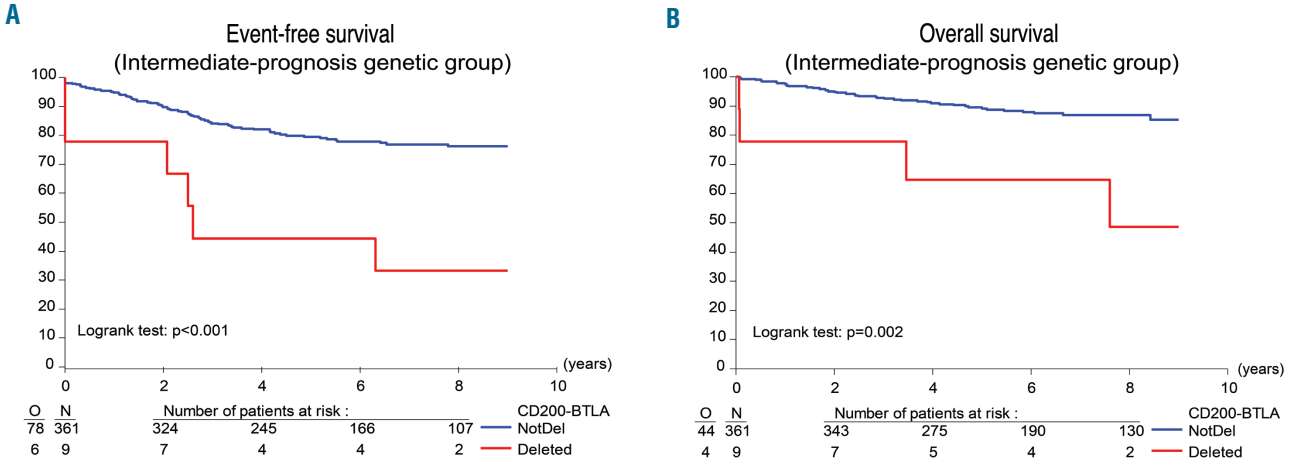

Figure 4. Associations between *CD200/BTLA* deletions and outcome in the intermediate-prognosis genetic risk group of BCP-ALL patients treated according to the EORTC 58951 trial. Kaplan-Meier curves of (A) event-free survival and (B) overall survival in the intermediate-prognosis genetic group according to the presence of *CD200/BTLA* deletions.

Table 4. Multivariate analysis of outcome in BCP-ALL patients enrolled in the EORTC-CLG 58951 trial.

		Event-free survival			Disease-free survival			Overall survival		
The whole cohort of patients		**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
CD200/BTLA Status	Deleted *vs.* non-deleted	1.98	1.18-3.32	0.01	1.84	1.06-3.19	0.03	1.73	0.79-3.78	0.17
IKZF1 status	Deleted *vs.* non-deleted	1.63	1.17-2.28	0.004	1.63	1.15-2.32	0.006	1.01	0.61-1.66	0.98
Gender	Male *vs.* female	1.67	1.24-2.27	<.001	1.80	1.31-2.47	<.001	1.18	0.78-1.78	0.44
Response to prephase	≥ *vs.* <1000 blasts/μL	1.58	1.02-2.46	0.04	1.16	0.70-1.94	0.57	2.00	1.13-3.53	0.02
Genetic status	Intermediate *vs.* good prognosis	2.36	1.68-3.31	<.001	2.35	1.65-3.34	<.001	3.21	1.95-5.28	<.001
Genetic status	Poor *vs.* good prognosis	4.96	3.17-7.75	<.001	5.59	3.55-8.82	<.001	6.35	3.36-12.01	<.001
The intermediate-prognosis genetic group		**HR**	**99% CI**	***P***	**HR**	**99% CI**	***P***	**HR**	**99% CI**	***P***
CD200/BTLA status	Deleted *vs.* non-deleted	3.00	0.97-9.31	0.01	2.11	0.54-8.29	0.16	4.31	1.06-17.54	0.007
IKZF1 status	Deleted *vs.* non-deleted	1.94	1.05-3.59	0.005	2.05	1.07-3.90	0.004	1.19	0.50-2.82	0.61
Gender	Male *vs.* female	2.07	1.14-3.74	0.002	2.36	1.25-4.47	<.001	1.77	0.82-3.83	0.06
Response to prephase	≥ *vs.* <1000 blasts/μL	1.49	0.68-3.29	0.19	0.97	0.36-2.56	0.93	1.40	0.48-4.08	0.42

in BCP-ALL disease pathology and that CD200 might represent the main functionally relevant target for this particular deletion.

Mapping of the exact genomic breakpoints of *CD200/BTLA* deletions in BCP-ALL suggests aberrant RAG-mediated recombination as the mechanism underlying this genetic alteration. Notably, a similar RAG "footprint" pattern was previously observed for recurring copy number alterations in BCP-ALL such as *PAX5*, *CDKN2A*, *BTG1*, *RAG1*, *RAG2*, *IKZF2* and *IKZF3*, *ERG*, *C20orf94*.[9,30]

In our study, we observed an association of *CD200/BTLA* deletions with inferior 8-year event-free survival (HR 2.02; 95% CI 1.23-3.32; *P*=0.005) and disease-free survival (HR 1.92; 95% CI 1.13-3.26; *P*=0.014) in BCP-ALL patients treated according to the EORTC-CLG 58951 protocol. Multivariate analysis confirmed the independent prognostic value of the *CD200/BTLA* deletions in the whole cohort of BCP-ALL patients. The lack of impact on overall survival indicates that relapsing patients can be rescued by second-line therapy. However, considering the increase in toxicity and late effects with increasing chemotherapy doses and the fact that many relapsing patients will eventually undergo hematopoietic stem cell transplantation, prevention of relapse by precise risk stratification remains an important issue. The timing of relapses (at/after the end of therapy) observed in this study, as well as the chemosensitivity to second-line rescue treatment, suggests that intensification of frontline treatment might prevent some of the relapses. Whether this should be achieved by treating patients at risk according to a high-risk schedule or by introducing less intensive modifications for instance during maintenance treatment, cannot be concluded yet.

CD200/BTLA deletions were most prevalent in the poor-prognosis genetic group (8/79=10.1%), but the highest number of *CD200/BTLA*-deleted cases was detected in the good-prognosis genetic group in which a strong association of *CD200/BTLA* deletions with *ETV6-RUNX1*-positive leukemias was observed (34 out of 56 deleted cases, *P*<0.0001). Notably, these findings further extend the causal role of aberrant RAG recombinase activity in the generation of cooperative genomic lesions that eventually lead to full leukemic transformation in *ETV6-RUNX1* ALL.[31] Moreover, the excellent clinical outcome of *ETV6-RUNX1* patients was not affected by the presence of *CD200/BTLA* deletions in this good-prognosis genetic subtype of BCP-ALL (*Online Supplementary Figure S2*). In contrast, although based on a small number of events, the presence of *CD200/BTLA* deletions in BCP-ALL patients within the intermediate-prognosis genetic group (9 out of 370) was associated with an inferior outcome.

In 2009, Mullighan and colleagues assessed the prognostic relevance of *IKZF1* alterations, *EBF1* and *CD200/BTLA* deletions in two independent cohorts with different sample composition and treatment schedules.[2] In the test cohort, all three genetic lesions were significantly associated with poor treatment outcome. However, this association could only be confirmed for *IKZF1* alterations in a validation series. Notably, the sample composition of the test and validation cohorts in this study was markedly different. In the test cohort, only three out of 221 BCP-ALL patients were positive for *ETV6-RUNX1*, whereas the majority of the patients could be allocated to the intermediate-prognosis genetic group due to the presence of the *TCF3-PBX1* translocation (25 out of 221) or the lack of any other known genetic prognostic marker (149 out of 221). Importantly, the sample distribution of the validation cohort was completely different, with *ETV6-RUNX1*-positive patients accounting for 19.3% (50 out of 258) of this cohort and only 34.5% (89 out of 258) being in the intermediate-prognosis genetic group. Given the strong link of *CD200/BTLA* deletions with prognostically favorable *ETV6-RUNX1* alterations and the association of *CD200/BTLA* deletions with poor outcome in the intermediate genetic risk group (this study), these differences in sample distribution might explain why the prognostic impact of *CD200/BTLA* deletions was not validated in this particular study.[2]

Overall, our study confirms that *CD200/BTLA* deletions are recurrent genetic lesions in the biology of BCP-ALL and identifies their exact frequency in a large and uniformly treated population of patients with BCP-ALL. *CD200/BTLA* deletions are highly enriched in *ETV6-RUNX1*-positive leukemias, suggesting a genetic interaction between these genomic alterations in the pathogenesis of BCP-ALL. Finally, our study demonstrates a prognostic value of *CD200/BTLA* deletions, probably restricted to a specific subgroup of BCP-ALL patients, and validates the previous association between *CD200/BTLA* deletions and poor clinical outcome in BCP-ALL.[2] CD200/BTLA deletions are not currently included in novel genetic risk classification algorithms in BCP-ALL, such as the one recently introduced by Moorman and colleagues.[32] However, our data suggest that *CD200/BTLA* deletions have potential value to be added to this algorithm, which is built by integrating data on copy number alterations from eight key genes/loci (*BTG1*, *PAX5*, *CDKN2A/B*, *IKZF1*, *ETV6*, *RB1*, *PAR1*, *EBF1*) with established cytogenetic risk groups.[32] Nevertheless, additional studies are mandatory to understand the exact role of *CD200/BTLA* deletions in malignant precursor B-cell transformation and their effects on treatment response.

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