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

Farzaneh Ghazavi,[1,2] Emmanuelle Clappier,[3,4] Tim Lammens,[1] Stefan Suciu,[5] Aurélie Caye,[3,4] Samira Zegrari,[3] Marleen Bakkus,[6] Nathalie Grardel,[7] Yves Benoit,[1] Yves Bertrand,[8] Odile Minckes,[9] Vitor Costa,[10] Alina Ferster,[11] Françoise Mazingue,[12] Geneviève Plat,[13] Emmanuel Plouvier,[14] Marilyne Poirée,[15] Anne Uyttebroeck,[16] Jutte van der Werff-ten Bosch,[17] Karima Yakouben,[18] Hetty Helsmoortel,[1,2] Magali Meul,[1] Nadine Van Roy,[2] Jan Philippé,[19] Frank Speleman,[2] Hélène Cavé,[3,4] Pieter Van Vlierberghe,[2,*] and Barbara De Moerloose[1,*]

[1]Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent University Hospital, Belgium; [2]Center for Medical Genetics, Ghent University Hospital, Belgium; [3]Department of Genetics, Robert Debré Hospital, APHP, Paris, France; [4]Hematology University Institute, University Paris-Diderot, Paris, France; [5]EORTC Headquarters, Brussels, Belgium; [6]Department of Hematology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel (VUB), Belgium; [7]Centre de Biologie Pathologie PM Degand, INSERM U837, Lille, France; [8]Institute of Hematology and Oncology Paediatrics, Hospices Civils de Lyon, France; [9]Department of Hematology, CHRU, Caen, France; [10]Department of Pediatrics, Portuguese Oncology Institute, Porto, Portugal; [11]Department of Hemato-Oncology, HUDERF, Brussels, Belgium; [12]Department of Pediatric Hematology-Oncology, CHR, Lille, France; [13]Department of Hematology, Children's Hospital, Toulouse, France; [14]Department of Hematolo-Oncology, CHRU, Besançon, France; [15]Department of Pediatric Onco-Hematology, Archet University Hospital, Nice, France; [16]Department of Pediatric Hematology-Oncology, University Hospitals Leuven, Belgium; [17]Department of Pediatrics, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel (VUB), Belgium; [18]Department of Pediatric Hematology, Robert Debré Hospital, APHP, Paris, France; and [19]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Belgium

**PVV and BDM contributed equally to this work.*

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Correspondence: barbara.demoerloose@uzgent.be

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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 (#NCT00002812). After obtaining informed consent from patients or their legal guardians according to the declaration of Helsinki, bone marrow (BM) and blood samples were taken at diagnosis, before 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 (CNS) prophylaxy without cranial irradiation, reinduction/late intensification and maintenance, and includes three randomised questions as described previously[1, 2].

Patients were assigned to different risk groups: very low-risk (VLR), average risk (AR) and very high-risk (VHR). VLR was defined as B-lineage ALL without high-risk criteria as mentioned before, with WBC counts below $10x10^9$/L, and with hyperdiploid karyotype (51-66 chromosomes) or DNA index >1.16 and <1.5, in the absence of CNS or gonadal involvement. VHR criteria consisted of blast count in peripheral blood ≥ $1x10^9$/L at completion of the prephase (day 8), presence of t(9;22), t(4;11) or another MLL rearrangement, near-haploidy (< 34 chromosomes), acute undifferentiated leukemia (AUL), MRD > 10^{-2} at completion of induction (Day 35) or failure to achieve complete remission (CR). AR patients were all children without VLR or VHR characteristics, and they were subdivided in AR1 and AR2 patient groups[1, 2].

Cell Culture

A panel of eight BCP-ALL cell lines were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) repository: REH, SEM, SUP-B15, NALM-6, NALM-16, RCH-ACV, HAL-01, 697. Cells were cultured in RPMI 1640 media (Life Technologies Europe) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Life Technologies Europe), 1% kanamycin (Life Technologies Europe), 1% glutamine (Life Technologies Europe) at 37° C in 5% CO2.

DNA Isolation

DNA of primary samples and cell lines was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For each sample, the concentration was measured using the NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA).

Array Comparative Genome Hybridization (array CGH)

The DNA of samples and cell lines was profiled on the 180K custom designed oligonucleotide array platform, enriched for genomic regions known to be recurrently implicated in leukemia and oligonucleotides encompassing non-coding RNAs (microRNAs and long non-coding RNAs). The array designs were based on the hg18 genome build. Utilizing random prime labeling (BioPrime ArrayCGH Genomic Labeling System, Invitrogen), 400 ng of tumor and control DNA (promega control DNA) was labeled with Cy3 and Cy5 dyes (Perkin Elmer, Waltham, MA, USA). Further processing was then performed according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA). Fluorescence intensities were measured using an Agilent scanner (G2505C, Agilent Technologies). Data were extracted using the Feature Extraction v10.1.1.1 software program (Agilent Technologies) and further processed with arrayCGHbase (http://medgen.ugent.be/arraycghbase)[3]. Regions of

copy number alteration were defined as a minimum of 3 adjacent clones simultaneously deviating beyond the threshold values (0.3 and -0.3).

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*. These arrays covered the following region on Chr3: 111,899,945-112,300,062 (hg19) with a total of 4314 probes. The generation of these arrays was performed using the online eArray tool from Agilent (https://earray.chem.agilent.com/suredesign/home.htm).

Mutation analysis

All exons of *BTLA* and *CD200* were amplified from genomic DNA using PCR. PCR primers are available in Supplementary Table 1. PCR reactions were carried out using KAPA Taq Hotstart PCR kit (KAPABiosystems, Wilmington, MA, USA). Briefly, 2 µl DNA (10ng/µl) was added to 23 µl of PCR mix (5µl 5X buffer, 2.5µl $Mgcl_2$ (25µM), 0.5µl dNTP (10mM), 0.2µl Taq (5U/µl), 1 µl Forward Primer (5µM), 1 µl Reverse Primer (5µM), 12.8 µl PCR-grade water). Direct sequencing of PCR products was performed through Sanger sequencing. The sequences were analyzed using Sequencher v4.7.

RNA isolation and RT-qPCR

Total RNA was isolated using the miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. For each sample, RNA concentration was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Nazareth Eke, Belgium) according to the instructions of the manufacturer. After cDNA preparation, RT-qPCR reactions were carried out using custom 2X SsoAdvanced SYBR Green Supermix (Bio-Rad). Briefly, 2 µl cDNA was added to 3 µl of PCR mix (2.5 µl 2X

SsoAdvanced mastermix, 0.25 µl Forward Primer (5µM), 0.25 µl Reverse Primer (5µM)). Relative expression values were calculated using the ΔCt-method. For normalization, the expression of at least three reference genes was combined to calculate a normalization factor.

Flow cytometry analysis of B cell surface expression of BTLA and CD200

Cell suspensions were incubated for 20 minutes with a cocktail of fluorochrome-conjugated antibodies. These included CD272(=BTLA)-PE, CD200-APC, CD3-FITC, CD19-PC7, CD10-APC-H7, CD20-V450 and CD45-V500. All antibodies were obtained from BD Biosciences (San Jose, CA, USA). Data were acquired on a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences) and analyses were performed with Infinicyt software version 1.7 from Cytognos (Salamanca, Spain). Gating was based on CD19 expression and scatter, after exclusion of doublets. The REH cell line (harboring a homozygous *CD200*/*BTLA* deletion) and isotype antibodies were used as control. Results were expressed as mean fluorescence intensity (MFI).

High throughput screening for *CD200/BTLA* deletions

Primers flanking the junctions were designed based on breakpoint sequences, using the Primer3Plus software and include:

F1: 5'- TTTCCGGAGTCTCAGAGAGGT -3',

F2: 5'- AGGCTTTGCTTCGTCTTCTG-3'

R(6-FAM-labelled): 5'- TTAAAGACGGCTGCTCTTCC -3'

Fifty nanograms of DNA were amplified using standard 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

EFS was calculated from the date of diagnosis to the date of last follow up or the first event (no CR, relapse or death). Disease-free survival (DFS) was calculated from the date of CR to the date of last follow up or the first event (relapse or death). All patients alive and in first CR were censored at their last follow-up. OS 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 according to the Kaplan-Meier technique, and standard errors of estimates were obtained via 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.

Reference:

1. Domenech c, Suciu S, De Moerloose B, et al. Dexamethasone (6 mg/m2/day) and prednisolone (60 mg/m2/day) were equally effective as induction therapy for childhood acute lymphoblastic leukemia in the EORTC CLG 58951 randomized trial. Haematologica. 2014;99(7):1220-1227.
2. De Moerloose B, Suciu S, Bertrand Y, et al. Improved outcome with pulses of vincristine and corticosteroids in continuation therapy of children with average risk acute lymphoblastic leukemia (ALL) and lymphoblastic non-Hodgkin lymphoma (NHL): report of the EORTC randomized phase 3 trial 58951. Blood. 2010;116(1):36-44.
3. Menten B, Pattyn F, De Preter K, et al. arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays. BMC bioinformatics. 2005;6(1):124.

Supplementary Table 1. Primers used for mutation analysis of *CD200* and *BTLA*

Gene	Coding exon	Forward primer sequence	Reverse primer sequence
CD200	1	ACACAGACAGCCTCCGCTC	TCAACACCGCTGATCTAGTCC
CD200	2	TTCCCTCCTCATATCCTGAGC	TTCATGCAGACAAATGGAACC
CD200	3	TTTCTCTGGCATCACGTAGGA	GGCACTACGGCTATATTCCAA
CD200	4	TGCCATGCTATCTTTCTAGCC	CAGGACATTCACTGCCTTCC
CD200	5	TGCGATAAGTTTAAAGCTCTTATTACAC	CACATTGTTGCCATTCCCTTA
CD200	6	CCCTCACACCATACTGGGTC	GATCCTTAAGGCTTTCGCTCC
BTLA	1	CTGCAGCACTCAGAAGACGA	GGAGAATGTTGCCTCCAAGA
BTLA	2	GCTTGAGAACTATTGAAGTCAC	CTATAGGAGTTGGCTTTCTAAC
BTLA	3	TGGCCTGAAATCTCCTTCAC	CAGGATTGGGAAAGAAAACAACAGG
BTLA	4	CCCTGGCATTCAACAGGATA	AATAATGCCTGGCACATGGT
BTLA	5	GCACTACCATGGCCGTAAGT	GACATCCTGTTGAGCCCAGA

Supplementary Table 2. Variations found in *CD200*, *BTLA* exons in 70 BCP-ALL patients

	rs no.
CD200	
5' upstream c.-77 G>C	-
c.12+32 G>T	(rs2276772)
c.12+49 G>A	(rs115847652)
c.12+102 T>C	(rs9883527)
p.Ser36Cys	(rs1131199)
c.170-104 T>C	(rs3817425)
c.170-44 G>A	(rs115358071)
p.Pro71Thr	(rs2272022)
c.497-45 C>T	(rs7612748)
p. Thr218=	(rs1050572)
c.770-119 C>T	(rs60377655)
c.770-65_770-64 insCTATTGCTTTATCA	(rs150687436)
c.770-56 C>T	(rs73227426)
c.770-42 C>T	(rs73227426)
c.877+19 T>A	(rs141526201)
BTLA	
p.Ser157Arg	(rs2931761)
p.Pro267Leu	(rs9288952)
c.*28T>C	(rs2171513)

*All variants are commonly reported SNPs, present within dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

Supplementary Table 3. Mean and median expression values of CD272 (BTLA) and CD200 in eight used B-ALL cell lines and 23 BCP-ALL patients.

	*Expression value of CD272 (Fluorescence intensity)		*Expression value of CD200 (Fluorescence intensity)	
	Mean	Median	Mean	Median
Cell lines				
697	17	12	1842	1566
REH	50	35	285	263
RCH-ACV	0	0	3332	2761
NALM-16	13	0	3332	2761
NALM-6	0	0	462	284
SEM	0	0	209	193
SUP-B15	0	0	1329	925
HAL-01	3	0	5564	4667
Deleted cases				
Patient 1	112	45	2144	1889
Patient 2	68	0	1337	1022
Patient 3	414	62	4998	3928
Wild type cases				
Patient 4	174	37	904	847
Patient 5	949	294	1786	1750
Patient 6	777	268	1406	1373
Patient 7	470	318	4671	3848
Patient 8	32	12	7976	7494
Patient 9	99	0	2836	2056
Patient 10	105	25	2733	2490
Patient 11	174	24	17564	15029
Patient 12	20	5	6475	5735
Patient 13	188	0	6449	5649
Patient 14	1017	37	11764	8105
Patient 15	623	15	9305	7543
Patient 16	342	42	7990	7342
Patient 17	142	0	6098	4421
Patient 18	156	17	12109	11017
Patient 19	180	21	6744	5560
Patient 20	147	50	12994	11720
Patient 21	746	88	3417	2126
Patient 22	905	681	7712	7255
Patient 23	328	0	3605	3020

* Data were acquired with FACSCanto II and analyses were performed with Infinicyt version 1.7. Events were gated on CD19 expression and scatter, after exclusion of doublets. CD19 was labelled with PE-Cy7, and CD200 with APC.

Supplementary Table 4: Clinical and biological features of BCP-ALL patients according to *CD200*/*BTLA* deletions status

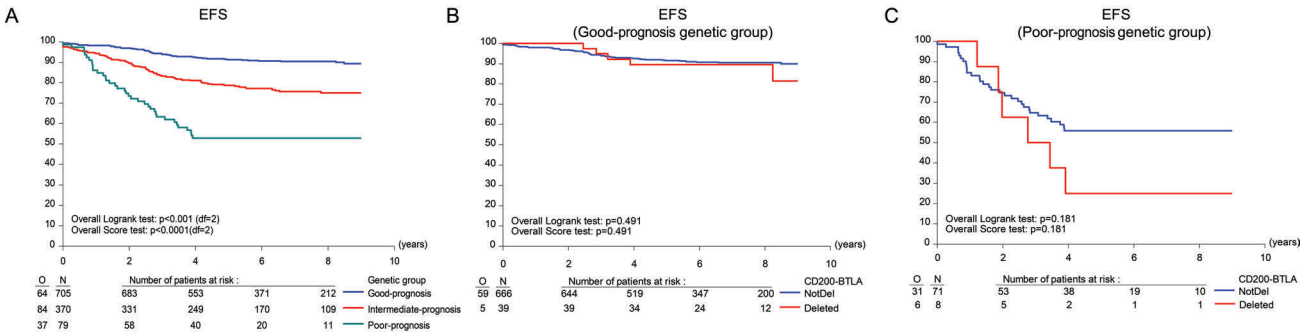
Characteristic	Not deleted (N=1098) No (%)	Deleted (N=56) No (%)	ND (N=98) No (%)
Gender:			
Male	587 (53.5)	30 (53.6)	45 (45.9)
Female	511 (46.5)	26 (46.4)	52 (53.1)
missing	0 (0.0)	0 (0.0)	1 (1.0)
Age at diagnosis (years):			
<1	3 (0.3)	0 (0)	0 (0.0)
1 < < 10	880 (80.2)	44 (78.6)	76 (78.6)
>=10	215 (19.6)	12 (21.4)	21 (21.4)
missing	0 (0.0)	0 (0.0)	1 (1.0)
WBC count (x10^9/L):			
<10	617 (56.2)	28 (50.0)	52 (53.1)
10-<25	214 (19.5)	15 (26.8)	21 (21.4)
25-<100	209 (19.0)	8 (14.3)	13 (13.3)
>=100	58 (5.3)	5 (8.9)	11 (11.2)
missing	0 (0.0)	0 (0.0)	1 (1.0)
NCI risk group:			
Standard Risk	772 (70.3)	37 (66.1)	62 (63.3)
High Risk	326 (29.7)	19 (33.9)	35 (35.7)
missing	0 (0.0)	0 (0.0)	1 (1.0)
Prephase response:			
<1x10^9/L	1027 (93.5)	53 (94.6)	95 (96.9)
>=1x10^9/L	71 (6.5)	3 (5.4)	1 (1.0)
missing	0 (0.0)	0 (0.0)	2 (2.0)
MRD after induction:			
< 10^{-2}	940 (85.6)	45 (80.4)	76 (77.6)
>= 10^{-2}	29 (2.65)	4 (7.1)	4 (4.1)
Not evaluable/missing	129 (11.75)	7 (12.5)	18 (18.4)
Initial CNS			
CNS-1	1028 (93.6)	54 (96.4)	89 (90.8)
CNS-2/TLP+	50 (4.6)	1 (1.8)	6 (6.1)
CNS-3	16 (1.5)	0 (0.0)	1 (1.0)
Missing	4 (0.4)	1 (1.8)	2 (2.0)
Genetic subtypes:			
BCR-ABL1	24 (2.2)	3 (5.4)	3 (3.1)
ERGdel	36 (3.3)	1 (1.8)	1 (1.0)
ETV6-RUNX1	248 (22.6)	34 (60.7)	23 (23.5)
High hyperdiploidy	382 (34.8)	4 (7.1)	34 (34.7)
Low hypo/near-hapl	9 (0.8)	1 (1.8)	1 (1.0)
MLL translocation	17 (1.5)	0 (0.0)	3 (3.1)
Other	314 (28.6)	9 (16.1)	29 (29.6)
TCF3-PBX1	47 (4.3)	0 (0.0)	2 (2.0)
iAMP21	21 (1.9)	4 (7.1)	2 (2.0)
IKZF1 Del	172 (15.7)	10 (17.9)	18 (18.4)
Genetic group:			
Good	666 (60.7)	39 (69.6)	58 (59.2)
Intermediate	361 (32.9)	9 (16.1)	31 (31.6)
High	71 (6.5)	8 (14.3)	9 (9.2)
EFS status			
NoCR	12 (1.1)	2 (3.6)	1 (1.0)
CCR	930 (84.7)	39 (69.6)	81 (82.7)
relapse	143 (13.0)	14 (25.0)	15 (15.3)
TRM	13 (1.2)	1 (1.8)	0 (0.0)
Missing	0 (0.0)	0 (0.0)	1 (1.0)
Survival Status			
alive	1012 (92.2)	49 (87.5)	88 (89.8)
dead	86 (7.8)	7 (12.5)	9 (9.2)
Missing	0 (0.0)	0 (0.0)	1 (1.0)

Abbreviation: ND, not determined; WBC, white blood cell; NCI, national cancer institute; MRD, minimal residual disease; CNS, central nervous system; TLP, traumatic lumbar puncture; CR, complete remission; CCR, continued complete remission; TRM, treatment related mortality.

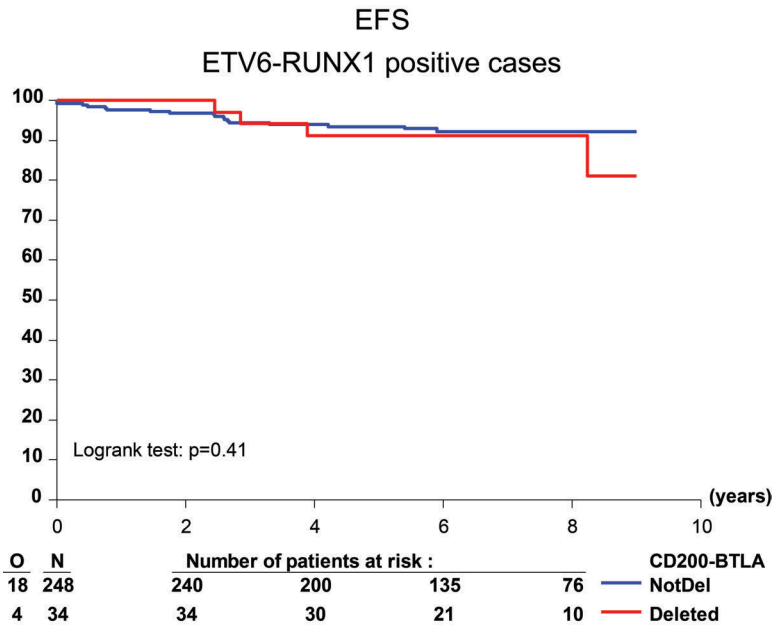
Supplementary Table 5. Clinical and biological features of intermediate-prognosis genetic group BCP-ALL patients according to *CD200*/*BTLA* deletions status

Characteristic	Not deleted (N=361) No (%)	Deleted (N=9) No (%)	P-value
Gender:			0.5
Male	181 (50.1)	3 (33.3)	
Female	180 (49.9)	6 (66.7)	
Age at diagnosis (years):			0.92
<1	2 (0.6)	0 (0.0)	
1 < < 10	244 (67.5)	6(66.7)	
>=10	115 (31.9)	3 (33.3)	
WBC count (x10^9/L):			0.25
<10	155(42.9)	5(55.5)	
10-<25	83 (23.0)	3 (33.3)	
25-<100	95 (26.3)	1 (11.1)	
>=100	28 (7.8)	0 (0.0)	
NCI risk group:			0.5
Standard Risk	193 (53.5)	6 (66.7)	
High Risk	168 (46.5)	3 (33.3)	
Prephase response:			0.54
<1x10^9/L	323 (89.5)	7 (77.8)	
>=1x10^9/L	38 (10.5)	2 (22.2)	
MRD after induction:			0.65
< 10^{-2}	293 (81.2)	6 (66.7)	
>= 10^{-2}	14 (3.9)	1 (11.1)	
Not evaluable/missing	54 (14.9)	2(22.2)	
Initial CNS			1
CNS-1	331 (91.7)	9 (100.0)	
CNS-2/TLP+	21 (5.8)	0 (0.0)	
CNS-3	9 (2.5)	0 (0.0)	
IKZF1			0.1
No	286 (79.2)	5 (55.6)	
Del	75 (20.8)	4 (44.4)	

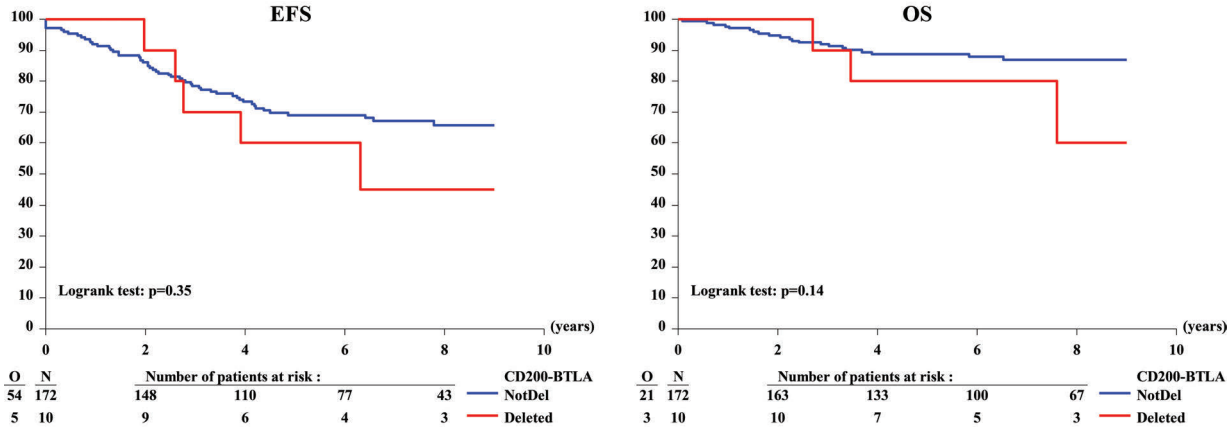
Abbreviation: WBC, white blood cell; NCI, national cancer institute; MRD, minimal residual disease; CNS, central nervous system; TLP, traumatic lumbar puncture.



Supplementary Figure 1: (A) Event-free survival (EFS) in all BCP-ALL patients allocated to three genetic risk groups. Associations between *CD200*/*BTLA* deletions and outcome (EFS) in (B) the good-prognosis genetic risk groups and (C) the poor-prognosis genetic risk groups of BCP-ALL patients treated according to the EORTC 58951 trial.



Supplementary Figure 2: *CD200*/*BTLA* deletions and event-free survival in *ETV6-RUNX1* positive BCP-ALL patients treated according to the EORTC 58951 trial.



Figures_Response_Reviewers: Association between *CD200/BTLA* deletions and (A) Event-free survival and (B) Overall survival in IKZF1 deleted subgroup of BCP-ALL patients treated according to the EORTC 58951 trial.