Copy number genome alterations are associated with treatment response and outcome in relapsed childhood *ETV6/RUNX1*-positive acute lymphoblastic leukemia

Almut Bokemeyer,[1] Cornelia Eckert,[1] Franziska Meyr,[1] Gabriele Koerner,[1] Arend von Stackelberg,[1] Reinhard Ullmann,[2] Seval Türkmen,[3,4] Günter Henze,[1] and Karl Seeger[1]

[1]Department of Pediatric Oncology/Hematology, Charité – Universitätsmedizin Berlin; [2]Max-Planck Institute for Molecular Genetics, Berlin; [3]Institute of Medical Genetics, Charité – Universitätsmedizin Berlin; and [4]Labor Berlin, Germany

ABSTRACT

The clinical heterogeneity among first relapses of childhood *ETV6/RUNX1*-positive acute lymphoblastic leukemia indicates that further genetic alterations in leukemic cells might affect the course of salvage therapy and be of prognostic relevance. To assess the incidence and prognostic relevance of additional copy number alterations at relapse of the disease, we performed whole genome array comparative genomic hybridization of leukemic cell DNA from 51 patients with first *ETV6/RUNX1*-positive relapse enrolled in and treated according to the relapse trials ALL-REZ of the Berlin-Frankfurt-Münster Study Group. Within this cohort of patients with relapsed *ETV6/RUNX1*-positive acute lymphoblastic leukemia, the largest analyzed for genome wide DNA copy number alterations to date, alterations were present in every *ETV6/RUNX1*-positive relapse and a high proportion of them occurred in recurrent overlapping chromosomal regions. Recurrent losses affected chromosomal regions 12p13, 6q21, 15q15.1, 9p21, 3p21, 5q and 3p14.2, whereas gains occurred in regions 21q22 and 12p. Loss of 12p13 including *CDKN1B* was associated with a shorter remission duration (P=0.009) and a lower probability of event-free survival (P=0.001). Distribution of X-chromosomal copy number alterations was gender-specific: whole X-chromosome loss occurred exclusively in females, gain of Xq only in males. Loss of the glucocorticoid receptor gene *NR3C1* (5q31.3) was associated with a poor response to induction treatment (P=0.003), possibly accounting for the adverse prognosis of some of the *ETV6/RUNX1*-positive relapses.

Introduction

Relapsed childhood acute lymphoblastic leukemia (ALL) is a clinically and biologically heterogeneous disease. Among patients with relapsed ALL, treatment response and outcome are dependent on the immunophenotype and on specific genetic aberrations in ALL cells, such as structural or numerical chromosome changes which characterize particular subtypes.[1] As in initial ALL, the chromosomal translocation t(12;21)(p13;q22) generating the fusion gene *ETV6/RUNX1* is the most common structural genetic aberration in pediatric B-cell precursor (BCP) ALL at first relapse.[2-7] Although generally associated with favorable risk features and advantageous long-term survival rates,[6,8] the high frequency at relapse of disease opposes this positive prognostic characteristic.[9,10]

In the current relapse trial of the Berlin-Frankfurt-Münster (BFM) study group, ALL-REZ BFM 2002, risk stratification and, accordingly, therapy intensity are based on well-established prognostic factors: *time of relapse* with respect to the cessation of frontline treatment, *site* of relapse (isolated or combined bone marrow or isolated extramedullary involvement), and *immunophenotype* (B- or T-lineage). Risk-adapted treatment of patients with first ALL relapse is based on these key prognostic determinants which are used to assign patients to three main risk groups with significantly different

event-free survival (EFS) rates.[11] Within the largest group, the intermediate-risk group, minimal residual disease assessment at the end of induction therapy is of strong prognostic relevance and is applied for stratification of post-remission therapy including further treatment intensification by stem cell transplantation.[12] Given the clinical characteristics of *ETV6/RUNX1*-positive ALL, such as *late* recurrence of the disease and BCP immunophenotype, most patients with this form of ALL are stratified into the intermediate risk group (*Online Supplementary Table S1*). Only 10% of *ETV6/RUNX1*-positive relapses occur *early*, and very early relapses are rare. A second continuous complete remission can be achieved in the majority of patients with *ETV6/RUNX1*-positive relapses. However, a considerable proportion exhibits a poor molecular response to treatment and eventually experiences a subsequent relapse. The underlying causes of the differences in response to treatment and subsequently outcome in the clinically heterogeneous group of *ETV6/RUNX1*-positive ALL relapses are not known. The current view is that despite the inability of the fusion oncoprotein ETV6/RUNX1 to induce leukemic transformation on its own, the *ETV6/RUNX1* fusion constitutes the initiating mutation in *ETV6/RUNX1*-positive leukemogenesis.[13-16] The identification of leukemia-initiating and -propagating cells in *ETV6/RUNX1*-positive ALL has recently corroborated this insight.[17] Secondary genetic alter-

ations are most likely required for *ETV6/RUNX1*-positive leukemogenesis and might be important for the differences in clinical outcome.

More than 80% of initial *ETV6/RUNX1*-positive ALL display additional genetic alterations of the *ETV6* and *RUNX1* gene loci in fluorescence *in situ* hybridization (FISH) analyses. These include deletions of the untranslocated *ETV6* gene (70%), an extra copy of *RUNX1* (23%) and duplication of the derivative chromosome der(21) t(12;21) (10%).[18] At relapse, additional alterations of chromosomes 12 and 21 are often detected in leukemic cells as well (85%).[19] Current genome wide high resolution analyses of DNA copy number alterations (CNA) have identified numerous genetic alterations in childhood ALL.[20-23] In initial *ETV6/RUNX1*-positive ALL, genes related to B-lymphocyte development and differentiation, cell cycle regulation, tumor suppression and apoptosis are recurrently affected.[20,21,24-27] Recent DNA copy number analyses on matched initial diagnosis and relapse ALL samples revealed that CNA acquired at relapse primarily affect genes implicated in cell cycle regulation, B-cell development, drug metabolism and drug response.[25,28,29] In the present study, leukemic cell DNA from 51 patients with a first relapse of *ETV6/RUNX1*-positive ALL enrolled in and treated according to the ALL-REZ BFM relapse trials were examined by whole genome array comparative genomic hybridization (CGH) for cooperating genetic lesions. This cohort represents the largest number of patients analyzed so far, enabling the investigation of an association between identified CNA and relapse-specific clinical and prognostic parameters for the first time.

Methods

Patients and samples

Leukemic bone marrow samples from 51 patients with first relapse of an *ETV6/RUNX1*-positive BCP-ALL were collected at relapse diagnosis after written informed consent was obtained from the patients, their parents or guardians in accordance with the ethical committee of the Charité and the declaration of Helsinki. All patients were enrolled in and treated according to ALL-REZ relapse trials 90, 95/96, and 2002 of the BFM study group, which were approved by the Institutional Review Boards of the Charité and the FU-Berlin, Germany. Diagnostic bone marrow samples were selected to contain >60% leukemic cells prior to further enrichment by Ficoll-density gradient separation of mononuclear cells. The presence of the *ETV6/RUNX1* fusion was detected by reverse transcriptase polymerase chain reaction analysis as described previously.[9]

DNA isolation

Leukemic cell samples were prepared and DNA isolated from bone marrow or peripheral blood mononuclear cells of all patients and of 20 healthy controls, serving as gender-specific control DNA, as described previously.[30] All DNA samples were amplified using the GenomePlex Whole Genome Amplification Kit (Sigma-Aldrich Chemie GmbH, Munich, Germany) following the manufacturer's directions.

Array comparative genomic hybridization

Array CGH was performed on a submegabase resolution whole genome tiling-path bacterial artificial chromosome (BAC) DNA array consisting of approximately 36,000 BAC clones obtained from several sources as described previously.[31] The array CGH data were visualized and analyzed using CGHPRO software (for details see the *Online Supplementary Information*).[32]

Fluorescence in situ hybridization

Array CGH findings of chromosome 5q31.3 (*NR3C1*), 6q21, and 12p13 (*CDKN1B*) were validated by interphase FISH on bone marrow smears obtained at relapse diagnosis. BAC clones RP11-D16614 (5q31.3; genomic position Mb 142.7-142.9) and RP11-M18773 (5p15.33; Mb 0.5-0.7; BACPAC Resources Center) were used as chromosome 5 probes and fluorescence-labeled with Vysis fluorophore-labeled dUTP (RP11-D16614: Spectrum Or-ange; RP11-M18773: FITC) by nick translation (Abbott, Wiesbaden, Germany). The cut-off value for deletion of *NR3C1* (7.78%) (5q31.3; RP11-D16614) was determined by interphase FISH analysis of blood smears from 20 healthy donors. For chromosome 6q21 and 12p13 findings, the 6q21 (SEC63)/SE6 probe (6q21: Spectrum Orange; SE6: Spectrum Green; Kreatech Diagnostics, Amsterdam, the Netherlands) and the BAC clone RP11-180M5 (12p13, Spectrum Orange, BlueGenome, Cambridge, UK) were applied, respectively. CEP 12 probe (D12Z1, Spectrum Green, Abbott) served as the control.

Statistical analysis

Statistical analysis was performed using STATA software (version 9.0, StataCorp, Texas, USA) and SPSS for Windows software (version 18.0.1, SPSS Inc., Chicago, USA). Associations between highly recurrent CNA (≥10% of relapses) and established prognostic determinants at ALL relapse (ALL-REZ BFM trials) were assessed by the Fisher exact and Mann-Whitney-U tests. Probabilities of event-free survival (pEFS) from the date of relapse diagnosis until the last recall or subsequent event (second relapse and death) were estimated by Kaplan-Meier analyses. Differences were considered by the log-rank test. To test the independence of predictive factors for pEFS, Cox regression analysis was performed by forward testing. The likelihood-ratio test was used for comparison of models. Due to the investigational nature of this analysis, we did not apply a stringent multiple comparisons ad-justment. However, all tests were conducted at the 1% significance level.

Results

Patients and representativeness

DNA from 51 leukemic bone marrow samples with a median blast count of 92% prior to enrichment (minimum 71%; maximum 98%) was hybridized to the tiling-path BAC array. Detailed clinical and genetic data are provided in *Online Supplementary Tables S1* and *S2*. The median follow-up time was 10.1 years (minimum 4.9; maximum 20.6). Comparison of the study cohort (n=51) with the total cohort of patients diagnosed with a first relapse of *ETV6/RUNX1*-positive BCP-ALL with bone marrow involvement enrolled in the ALL-REZ BFM relapse trials over the same period (n=113) confirmed the representativeness of the patients included (*Online Supplementary Table S1*).

Copy number alterations are a common feature of relapsed ETV6/RUNX1-positive acute lymphoblastic leukemia

Every relapse sample exhibited at least one additional chromosomal aberration. Chromosomal losses were approximately 2.5-fold more frequent than chromosomal gains and generally affected smaller genomic regions (Figure 1).

Whole chromosome gains were observed in 12 relapses, nine of which displayed a whole chromosome gain of a single chromosome only. One relapse exhibited a hyperdiploid karyotype (+10, +16, +18, +21). Overall +10 was the most frequent whole chromosome gain (6/51). Whole chromosome losses were detected in 12 relapses: the most common whole chromosome loss was that of chromosome X (9/51) (*Online Supplementary Table S3*).

Recurrently altered regions at relapse and their clinical association

A substantial number of CNA in relapsed *ETV6/RUNX1*-positive ALL occurred in recurrently affected regions. About 65% of detected CNA were identified in regions that were affected in at least two of the analyzed relapses, and 48% in more than five relapses which were, therefore, considered as highly recurrent (Table 1).

Deletions of chromosomal region 12p13 (49% of relapses) varied in size ranging from loss of large parts of chromosome 12p to microdeletions of only 0.31 Mb, limited to *ETV6* (22/51, 43%) (Table 1). *BCL2L14* and *CDKN1B*, located adjacent and approximately 0.8 Mb centromeric to

ETV6, respectively, were deleted in 23/51 (45%) and 19/51 (37%) relapses. The minimal overlapping region of loss including *CDKN1B* and *BCL2L14* (Table 1) contained several genes except for *ETV6*. A subset of 16 relapses showed losses including *CDKN1B*, *BCL2L14* and *ETV6*. FISH validation of the 12p13 findings (*Online Supplementary Figure S1*) confirmed the deletion in all of the investigated samples. Bone marrow smears were available from 12/19 (63%) of the relapsed patients with loss of the minimal overlapping region on chromosome 12p13 including *CDKN1B* among other genes. All of the deletions were heterozygous and detectable in 64-99% of screened nuclei (*Online Supplementary Table S6*). Analysis of the clinical data revealed that copy number losses including *CDKN1B* (37%), were significantly associated with a shorter first remission ($P=0.009$) and with inferior pEFS ($P=0.001$) and probability of overall survival ($P<0.001$) (Table 2). Patients with loss of this region had a pEFS ($\pm$ SE) at 10 years of 42% $\pm$ 11% compared to 81% $\pm$ 7% for those without loss (Figure 2; Table 2). Independence of the association of losses at 12p13 including *CDKN1B* and *BCL2L14* with the pEFS was tested by
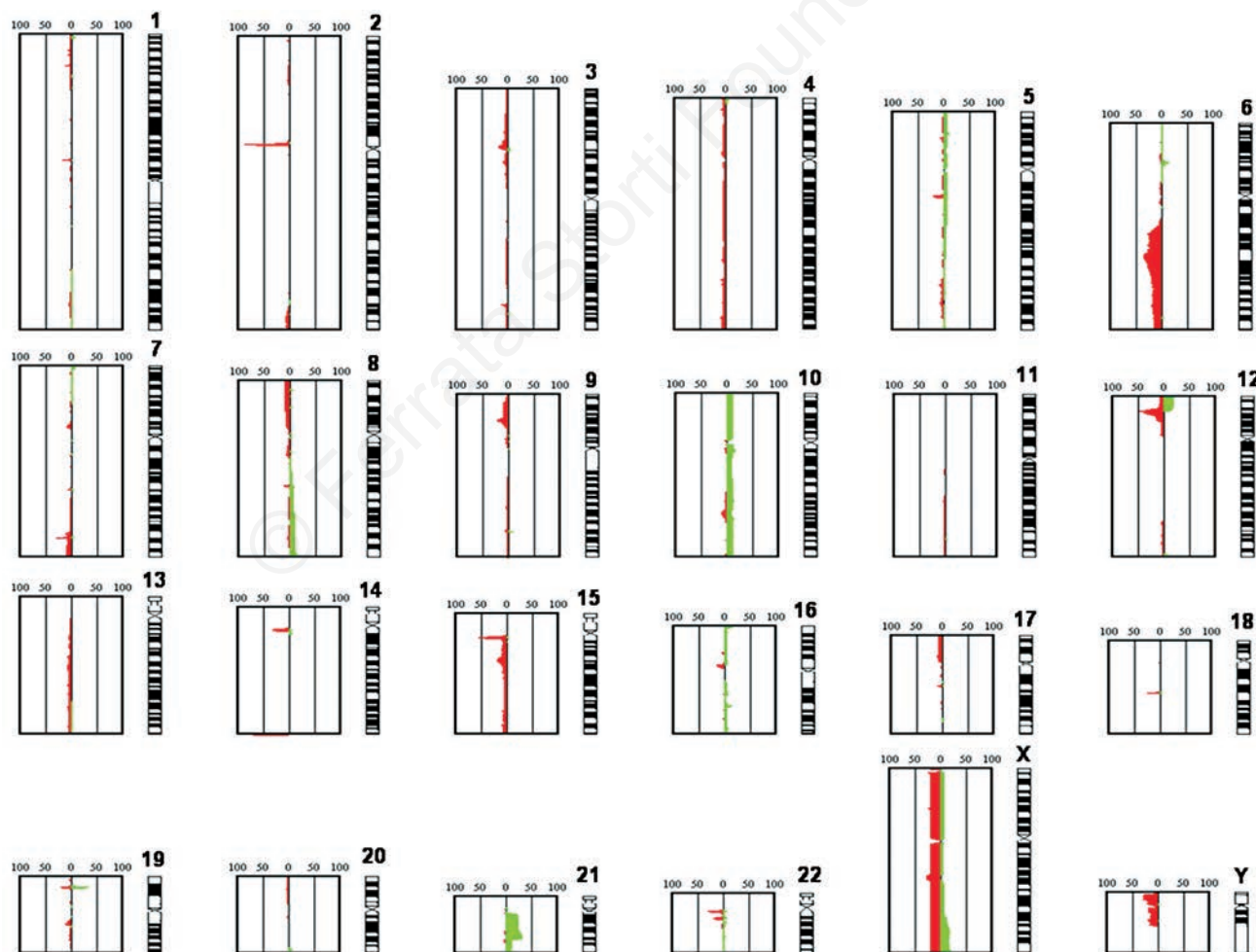

Figure 1. Summation of the total of identified CNA in 51 *ETV6/RUNX1*-positive ALL relapses. The figure shows the relative frequency of CNA identified in all the investigated relapses. Copy number losses are indicated in red, copy number gains in green. The numbering 0-50-100 indicates the number of relapses showing the CNA. The diagram is based on an automatic readout of the array CGH data without a correction of the data regarding copy number variants.

multivariate Cox regression analysis testing all relevant parameters. The final model included time of relapse as the strongest prognostic variable, the immunophenotype and loss of 12p13 including *CDKN1B* and *BCL2L14* showing a weaker prognostic impact (hazard ratio 2.28, 95% CI: 0.95-19.47, P=0.059) than in the univariate analysis (hazard ratio 4.60, 95% CI: 1.69-12.50, P=0.003) (*Online Supplementary Table S4*).

The second most frequent alteration was loss of 6q21 (17/51, 33%). Confirmation of the deletion by interphase FISH (*Online Supplementary Figure S1*) verified heterozygous deletions in all of the investigated samples (bone marrow smears obtainable in 8/17 cases, 47%) (*Online Supplementary Table S7*). The commonly deleted region (Table 1) contained multiple genes, including *FOXO3A, AIM1, ARMC2* and *SESN1*. The protein tyrosine kinase *FYN* was involved in 31% of 6q21 losses. Concomitant losses of 6q21 and 12p13.2 were noticeably frequent in the analyzed relapse samples (12/51, 24%) and included *CDKN1B* (12p13.2) in 16% (8/51) of cases. This subgroup was characterized by a high incidence of subsequent relapses (5/8, 63%). Statistical analysis showed a tendency towards both an inferior outcome (P=0.047) and an inferior pEFS (P=0.045) which was not attributable to the combined loss of 6q21 with *ETV6* (10/51, 20%) (Table 2;

Online Supplementary Figure S2). Since loss of 6q21 and *CDKN1B* were among the most frequent CNA, statistical analysis was done to assess whether the occurrence of both was associated. The comparison by cross-classified tables (Fisher exact test) showed no statistically significant association.

The commonly deleted region of 9p21.3 (11/51, 22%; Table 1) comprises 19 genes including *CDKN2A* (encoding p16[INK4A] and p14[ARF]), *CDKN2B* (p15[INK4B]) and *MTAP* (methylthioadenosine phosphorylase). Patients displaying loss of 9p21.3 in their leukemic cells had higher peripheral blast cell counts (P=0.002), higher leukocyte counts (P=0.001) and were older (P=0.0005) at relapse diagnosis.

Chromosomal gains mainly involved chromosomes 12 and 21. The most frequently gained region was 21q22 (17/51, 33%) followed by gain of 12p (22%). Gains of 12p were always accompanied by gain of 21q, a finding compatible with the existence of an extra der(21)t(12;21).

Copy number alterations affecting genes associated with B-lymphocyte differentiation

Several of the identified copy number losses affected genes associated with B-lymphocyte development and differentiation. These included *IKZF1* and *PAX5* (4/51, 8%), the early B-cell factor gene *EBF1* (3/51, 6%) and the

Table 1. Recurrent CNA in relapsed childhood *ETV6/RUNX1*-positive ALL.

Chr[1]	Cytoband	Start MOR[2] (Mb)	End MOR[2] (Mb)	Size MOR[2] (Mb)	N. of cases(%)		N. of genes within MOR[2]	Selection of candidate genes
Copy number losses								
3	p14.2	59.98	60.55	0.57	6	(12)	1	*FHIT*
3	p21.21	47.18	48.04	0.68	7	(14)	11	*SMARCC1, MAP4*
3	q26.32	178.38	178.73	0.35	6	(12)	1	*TBL1XR1*
4	q31.23-31.3	149.81	150.28	0.47	4	(8)	no annotated gene	
5	q33.3	157.2	158.5	1.3	3	6)	3	*EBF1*
5	q31.3	142.70	143.11	0.41	5	(10)	1	*NR3C1*
6	q21	108.00	111.00	3.20	17	(33)	26	*FOXO3A, AIM1, ARMC2, SESN1*
7	p12.1-12.2	49.87	50.93	1.06	4	(8)	6	*IKZF1*
7	q34	138.31	138.32	0.01	7	(14)	1	*TTC26*
9	p13.2	36.92	37.30	0.27	4	(8)	3	*PAX5, ZCCHC7*
9	p21	21.07	22.29	1.21	11	(22)	23	*CDKN2A, CDKN2B, MTAP*
10	q24.1	97.58	98.66	1.08	5	(10)	15	*BLNK (SLP-65)*
12	p13.2	11.69	12.00	0.31	22	(43)	1	*ETV6*
12	p13.2	11.94	13.20	1.26	19	(37)	22	*CDKN1B, BCL2L14*
15	q15.1	39.39	39.88	0.49	10	(20)	11	*LTK, ITPKA, TY-RO3*
19	q13.11	38.97	39.71	0.73	6	(12)	7	*GPI, PDCD2L, UBA2, WTIP*
Copy number gains								
3	p21.21	50.26	50.72	0.50	4	(8)	21	*CISH, HEMK1, RASSF1, HYAL2*
8	q23.1-24.3	108.98	143.22	34.24	5	(10)	multiple	*cMYC*
9	p13.3	34.42	34.66	0.24	5	(10)	12	*OPRS1, IL11RA, CCL27*
12	p13.2-13.33	0.01	12.00	11.98	11	(22)	multiple	*ETV6 in 7/11*
16	q22.1	65.72	66.24	0.52	8	(16)	26	*HSF4, E24F, CTCF, FAM65A*
21	q21.30-22.12	31.75	35.20	3.40	17	(33)	45	*RUNX1*
X	q27.3-28	144.07	154.63	10.56	7	(14)	multiple	*SPANX1*

[1]Chr: chromosome; [2]MOR: minimal overlapping region. Recurrent whole chromosome aneuploidies as well as recurrent CNA most likely arising from B- and T-cell antigen receptor gene rearrangements are excluded.

B-cell differentiation regulator *SLP-65* (*BLNK*) (5/51, 10%) (Table 1).

X-chromosomal copy number alterations show a gender-specific distribution

As mentioned above, the most frequently detected aberration affecting an entire chromosome was a whole chromosome loss of the X-chromosome (9/51, 18%). This loss was identified exclusively in female patients (*P*<0.0001) and was associated with older age at relapse diagnosis (*P*=0.006).

In contrast to whole chromosome loss of the X chromosome, a gain of Xq (7/51, 14%) was observed exclusively in male patients. As regards the clinical features of patients displaying gain of Xq, all were found to be in continuous complete remission.

Loss of 5q31.3 is associated with poor response to treatment

The extent of loss of 5q31.3 (5/51, 10%) varied from a large deletion to submicroscopic deletions in most samples (Table 3). The minimal overlapping region of 0.4 Mb (Table 1) contained exclusively the glucocorticoid receptor gene *NR3C1*. The 5q31.3 deletion was confirmed by FISH analyses on bone marrow smears from relapse diagnosis (*Online Supplementary Figure S1*). The proportion of cells exhibiting a deletion of *NR3C1* varied between 38% and 74%. Although heterozygous deletions were more frequent than homozygous deletions, the latter occurred in up to 17% of screened nuclei (*Online Supplementary Table S5*). Statistical evaluation of the clinical data of the five patients with loss of *NR3C1* in their leukemic cells (Table 3) revealed both a significantly worse molecular response to induction treatment (*P*=0.004) and a delayed morphological response (*P*=0.009) compared to *ETV6/RUNX1*-positive relapses without loss of *NR3C1*. Although molecular response status (minimal residual disease) was not known for two of the five patients, morphological evaluation revealed that these patients had not achieved remission after induction treatment (≥5% leukemic blasts). Both patients were, therefore, classified as molecular poor responders for the statistical analysis. Regarding the clinical course of disease in the five patients with loss of *NR3C1* (Table 3), three experienced a subsequent relapse and eventually died. Two patients underwent matched sibling donor hematopoietic stem cell transplantation in second remission and are in continuous complete remission for 8 and 9 years.

Table 2. Detailed clinical and prognostic characteristics of the patients with loss of chromosome 12p13.2, including *CDKN1B* and *BCL2L14*, of chromosome 6q21, and combined loss of 6q21 and *ETV6*.

Parameter	12p13.2 including CDKN1B and BCL2L14					6q21					6q21 and *ETV6*				
	not deleted		deleted		P value	not deleted		deleted		P value	not deleted		deleted		P value
	N.	%	N.	%		N.	%	N.	%		N.	%	N.	%	
N./percentage of patients	32	63	19	37		34	67	17	33		41	80	10	20	
Gender					0.074					0.42					0.10
male	16	50	15	79		22	65	9	53		25	61	6	60	
female	16	50	4	21		12	35	8	47		16	39	4	40	
Time of relapse					0.009					0.46					1.00
early	2	6	7	37		5	15	4	24		7	17	2	20	
late	30	94	12	63		29	85	13	76		34	83	8	80	
Duration of first remission					0.041					0.86					0.6
median (95%CI)	9	5-11	2	1-10		9	5-10	8	2-11		9	5-10	8	80	
Site of relapse					0.73					0.3					0.43
BM isolated	26	81	14	74		25	74	15	88		31	76	9	90	
BM combined	6	19	5	26		9	26	2	12		10	24	1	10	
Immunophenotype					0.018					1.00					1.00
common ALL	20	63	18	95		25	74	13	76		30	73	8	80	
pre-B ALL	12	37	1	5		9	26	4	24		11	27	2	20	
Risk group					0.18					1.00					1.00
intermediate	30	94	15	79		30	88	15	88		36	88	9	90	
high risk	2	6	4	21		4	12	2	12		5	12	1	10	
PBC (/µL)					0.046					0.8					0.97
median (95%CI)	381	266-928	2981	615-6808		612	315-2358	1150	194-4401		680	356-1809	787	95-16701	
MRD response after induction					0.47					0.15					0.21
good	14	58	8	73		13	54	9	82		15	56	7	88	
poor	10	42	3	27		11	46	2	18		12	44	1	12	
unknown	8		8			10		6			14		2		
pEFS at 15 years (±SE)					0.001					0.45					0.3
	0.81	0.069	0.42	0.11		0.71	0.078	0.59	0.12		0.71	0.071	0.5	0.16	

PBC: peripheral blast count; BM: bone marrow; MRD: minimal residual disease; pEFS: probability of event-free survival; SE: standard error; [1]Early: later than 18 months after initial diagnosis but before 6 months after cessation of frontline treatment; late: later than 6 months after cessation of frontline treatment. [2]Risk group stratification according to the ALL-REZ BFM protocols. [3]MRD good: one residual leukemic cell among more than 1000 normal cells (<10^3) after two induction courses; MRD poor: more than one residual leukemic cell among 1000 normal cells (>10^3) after two induction courses.

Discussion

This genome-wide study on *ETV6/RUNX1*-positive leukemic cell samples at first relapse was aimed at identifying CNA that possibly not only contribute to leukemic transformation of this leukemic subtype but also influence response to relapse therapy and subsequent recurrence of disease. A representative, large cohort of patients was analyzed. The results indicate that *ETV6/RUNX1*-positive ALL relapses are hallmarked by further numerical genomic alterations and that some of the identified CNA show an association with clinical characteristics.

The finding that chromosomal losses clearly outnumber gains and generally involve smaller genomic regions is consistent with the results on *ETV6/RUNX1*-positive ALL at first presentation.[20,21,25] In addition, recent studies showed an increase in the number of CNA at relapse.[25,28,29] The two most frequent CNA were losses of the chromosome bands 12p13 (49%) and 6q21 (33%). Loss of 12p13, including *ETV6*, is a known frequent alteration in initial ALL.[5] In initial *ETV6/RUNX1*-positive ALL, loss of *ETV6* has been described in 42 - 70% of cases.[21,25,26] At relapse, a recent study found *ETV6* losses in 57% of *ETV6/RUNX1*-positive ALL,[25] a proportion which coincides with a previous interphase FISH-based analysis by our group in which *ETV6* losses were detected in 52% of relapses[19] and with the present study. Further candidate genes affected by losses at 12p13 were *BCL2L14*, encoding a pro-apoptotic member of the Bcl-2 family,[33] and *CDKN1B*, coding for a cyclin-dependent kinase inhibitor, a negative regulator of

cell cycle progression from G1 to S phase.[34] Thirty-seven percent of relapses showed losses of the minimal overlapping region including these genes. Losses involving *CDKN1B* and *BCL2L14*, among other genes, were associated with significantly shorter remissions and inferior pEFS and probability of OS. However, multivariate analysis did not detect an independent prognostic influence and revealed that the association with pEFS was confounded by the time of relapse diagnosis and immunophenotype. Heterozygous deletions of *CDKN1B* have been recurrently described in initial ALL and have frequently been detected in *ETV6/RUNX1*-positive samples.[35] Though an association with *CDKN1B* gene deletion and a dismal prognosis has not been described in ALL, lower expression of *CDKN1B* has been shown to correlate with a poor prognosis in solid tumors.[36,37]

Numerical and structural aberrations involving chromosome 6q are common in childhood ALL.[38] In our relapsed *ETV6/RUNX1*-positive cohort, the rate of 6q21 losses (33%) was clearly higher than the reported incidence in initial disease (7-18%).[20,21,24,25] This frequency exceeds the occurrence of reported 6q losses in relapsed ALL in general (17%)[39] and of 6q21 losses in *ETV6/RUNX1*-positive relapses in particular (7%).[25]

In line with previous studies assessing the association between loss of 6q and clinical features in initial ALL,[38,40] no prognostic impact was found in the present study on *ETV6/RUNX1*-positive relapses. However, the combined loss of 12p13 (including *CDKN1B* and *BCL2L14*, among other genes) and 6q21 was frequent in the analyzed
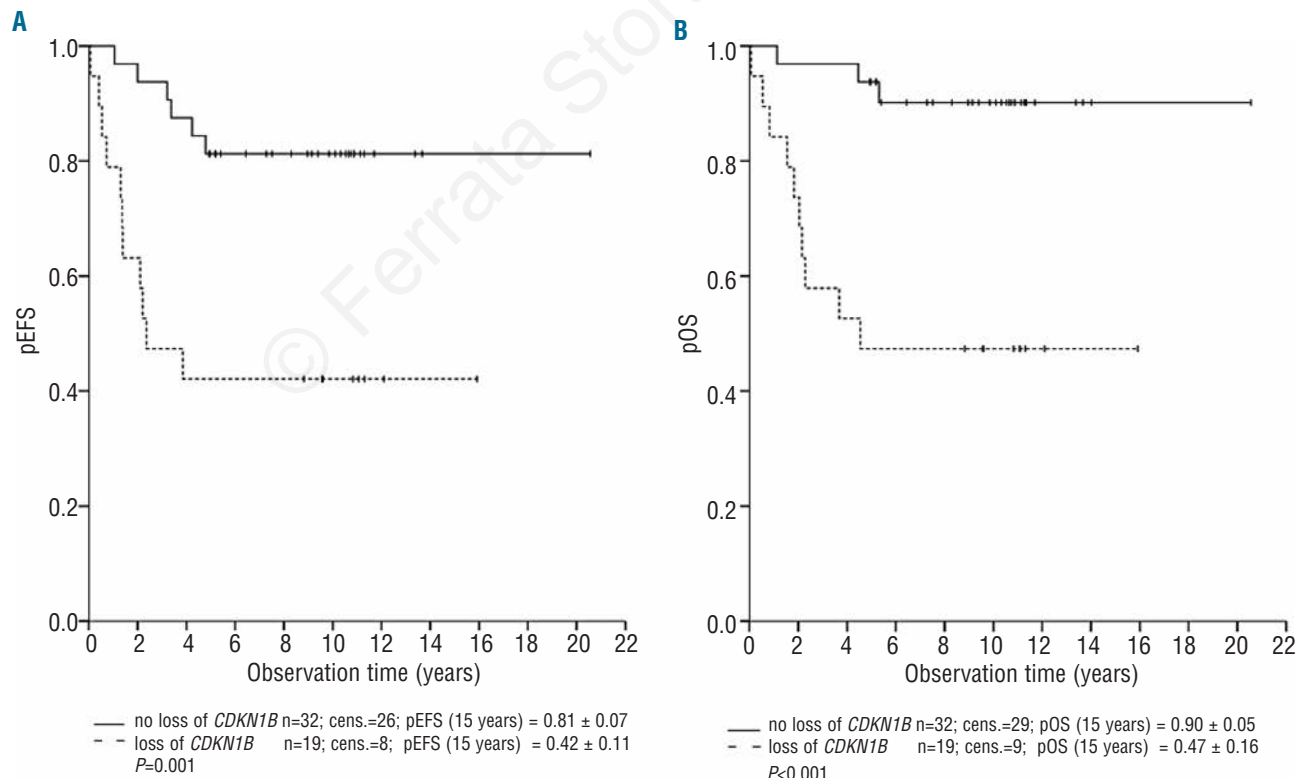

Figure 2. Probability of event-free survival (pEFS) and overall survival (pOS) (± standard error) as a function of CNA of chromosome region 12p13 including *CDKN1B*. pEFS (A) and pOS (B) differ significantly between the two groups defined by *CDKN1B* status (*P*=0.001, and *P*<0.001, respectively). The pEFS is 42% (±11%) and the pOS is 47 (±16%) in the group of patients with loss of this region compared to 81%±7% (pEFS) and 90%±5% in the group without loss of the region.

Table 3. Clinical and genetic characteristics of ALL relapses with loss of 5q31.3.

Patient ID	Clinical data		Start and end positions, and genomic size of deletions			
	Therapy response[1]	Course of disease	Start (Mb)	End (Mb)	Size (Mb)	Gene(s) in region
P19	Poor[2]	Second relapse, deceased	142 706	143 652	0.95	*NR3C1* (exons 1,2) 3 others
P26	Poor[2]	Second relapse after MUD-SCT, deceased	140 643	145 578	4.93	*NR3C1* and several others
P41	Poor[2]	Second relapse after MUD-SCT, deceased after third relapse	142 629	143 119	0.48	*NR3C1*
P44	Poor[2]	In CCR after MSD-SCT	142 629	143 179	0.55	*NR3C1* and 1 other
P46	Poor[2]	In CCR after MSD-SCT	138 368	170 518	32.15	*NR3C1* and multiple others

SCT: stem cell transplantation; MUD-SCT: matched unrelated donor-SCT; MSD-SCT: matched sibling donor-SCT; CCR: continuous complete remission; [1]Response to therapy after two induction courses as assessed by minimal residual disease (P19, P44, P46) or morphological remission (P26, P41). [2]Poor: more than one residual leukemic cell among 1000 normal cells or 5-25% (P26) and more than 25% (P41) leukemic cells in the bone marrow after induction therapy.

relapses (8/51, 16%). Subsequent relapses were disproportionately more frequent among patients with this combination of losses than in those without (subsequent relapses in 63% *versus* 24%), which was also reflected by an inferior pEFS. Since both of the identified regions of overlap were relatively large (3.2 Mb, 6q21; 1.26 Mb, 12p13) and included several genes, the observed association with a dismal prognosis is difficult to interpret. Nonetheless, it can be presumed that genes affected by the deletion are essential for normal cellular homeostasis. FOXO3A, a transcription factor involved in the control of proliferation and apoptosis as well as in the induction of *CDKN1B* gene transcription,[41] was included in the deleted region at 6q21. Thus co-inactivation of both FOXO3A (6q21) and CDKN1B (12p13) caused by copy number losses might have an adverse prognostic impact.

The incidence of deletions at 9p21.3 (22%), including *CDKN2A*, *MTAP* and *CDKN2B*, in initial *ETV6/RUNX1*-positive ALL varies between 12% and 29%.[20,21,24] In our relapsed cohort (22%), a significant association was seen between loss of 9p21.3 and higher peripheral blast cell and leukocyte counts as well as older age at relapse diagnosis. Consistent with other studies which analyzed childhood BCP-ALL, no correlation was observed between loss of this region and the outcome of patients.[42,43]

Involvement of B-cell differentiation genes

Recently, several studies reported recurrent CNA of genes associated with B-lymphocyte differentiation in ALL at initial presentation.[21,22,29] These genes included the B-lineage transcription factors *PAX5*, *EBF1* and *IKZF1*, as well as genes with other established functions in B-cell development such as *FYN*, *RAG1* and *RAG2*. Several of these genes were affected by CNA in *ETV6/RUNX1*-positive relapsed ALL as well, but mostly at lower frequencies than described before. Loss of *PAX5* was only detected in 8% of the analyzed samples, a proportion clearly lower than those found by Tzusuki *et al.*,[24] Mullighan *et al.*,[21] and Lilljebjörn *et al.*[26] at first presentation (6/24, 25%; 13/47, 27%; 6/24, 25%, respectively) and at relapse of *ETV6/RUNX1*-positive ALL (3/14, 21%).[25]

Two recent studies compared the incidence of CNA in paired initial and relapsed BCP-ALL[29] and *ETV6/RUNX1*-positive ALL[25] samples. *PAX5* losses were observed at both stages of disease in 35%[29] and 21%,[25] respectively. In the comparative analysis of 14 paired *ETV6/RUNX1*-positive ALL samples,[25] acquired losses of *EBF1* (2/14) and *IKZF1*

(1/14) were detected at relapse. In our larger cohort of 51 relapsed *ETV6/RUNX1*-positive ALL, *EBF1* and *IKZF1* deletions occurred in 6% and 8% of cases, respectively. Deletions of *IKZF1* have also been reported at initial presentation of *ETV6/RUNX1*-positive ALL, albeit at a lower frequency.[21,26,44] However, *IKZF1* is frequently deleted in *BCR/ABL*-positive[45] and other high-risk BCP-ALL subtypes.[46]

X-chromosome copy number alterations

CNA of the X-chromosome showed a gender-specific distribution at relapse. Loss of the whole X-chromosome was the most frequent whole chromosome aneuploidy (18% in the entire cohort) and was confined to female patients (9/20 females, 45%). Previous cytogenetic studies found a lower incidence of whole chromosome loss of X (3-8%) at initial presentation of ALL,[47,48] though an association with female gender and *ETV6/RUNX1*-positivity was observed as well. In contrast, gain of Xq (14% of relapses) was restricted to male patients (13/31, 42%). Gain of Xq has been described as one of the most frequent CNA in initial *ETV6/RUNX1*-positive ALL (6/17, 35%), occurring exclusively in ALL samples from male patients.[20] Consistent with our results, gain of Xq constantly included the telomere of Xq while the centromeric breakpoints affected variable regions of the long arm. Borst *et al.*[27] detected gain of Xq in 11% (six males, one female) and Kawamata *et al.*[23] in 2% of *ETV6/RUNX1*-positive ALL at first presentation.

Loss of glucocorticoid receptor gene NR3C1

Most significantly, loss of 5q31.3 (10% of *ETV6/RUNX1*-positive ALL relapses) was associated with an adverse impact on response to treatment. The *NR3C1* gene encoding the human glucocorticoid receptor was the single gene identified within the minimal overlapping region of loss. Confirmation by BAC-based FISH analyses detected homozygous as well as heterozygous deletions in the five relapses. Since glucocorticoids represent an essential component in frontline and relapse treatment protocols for ALL, we hypothesize that the poor treatment response of the five patients with loss of 5q31.3 might have originated from their *NR3C1* gene deletions.

Resistance to glucocorticoids is frequently observed clinically at relapse, and *in vitro* sensitivity assays show that leukemic cells from patients with relapsed ALL are much more resistant to glucocorticoids than at first pres-

entation.[49] However, although cell line models implicate mutations and loss of heterozygosity of the glucocorticoid receptor gene as mechanisms of *in vitro* glucocorticoid resistance,[50-52] results from mutational screening studies suggest that the contribution of somatic mutations to glucocorticoid resistance is rather negligible in initial and relapsed ALL patients.[51,53,54] In initial ALL, the frequency of *NR3C1* loss was approximately 10% in *ETV6/RUNX1*-positive ALL but considerably lower in *ETV6/RUNX*-negative cases.[21,26] The analysis of paired initial and relapse bone marrow samples showed *NR3C1* deletions were acquired at relapse in 9% of BCP-ALL relapses[28] and in 21% of *ETV6/RUNX1*-positive relapses.[25]

In our study, patients with *NR3C1* loss displayed clinical features distinct from those of the majority of patients with *ETV6/RUNX1*-positive relapses. None of the patients with loss of *NR3C1* had achieved molecular remission at the end of induction therapy at relapse and, therefore, received further treatment intensification with hematopoietic stem cell transplantation. Consideration of the course of disease demonstrates the benefit of risk stratification by minimal residual disease as well as the need for further biological risk markers that enable a more detailed understanding of poor treatment response and possibly help to rationalize specific treatment intensification before stem cell transplantation in poor responding *ETV6/RUNX1*-positive relapses.

In summary, array CGH analysis of relapsed childhood *ETV6/RUNX1*-positive leukemic cells allowed the identification of various highly recurrent CNA. The correlation with clinical characteristics revealed a prognostic relevance for several of the detected alterations. Most significantly, array CGH enabled the molecular characterization of subgroups with inferior outcomes and poor cytological/molecular response to induction treatment. The results of this study underscore the clinical and prognostic relevance of additional CNA in *ETV6/RUNX1*-positive ALL and constitute a basis for further analyses of the underlying molecular mechanisms that might contribute to the clinical heterogeneity within the large subgroup of *ETV6/RUNX1*-positive relapses. Prospectively, high resolution genomic profiling will yield valuable information for further improvement of risk stratification to provide more refined and individualized therapy in relapsed childhood ALL.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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