Genes commonly deleted in childhood B-cell precursor acute lymphoblastic leukemia: association with cytogenetics and clinical features

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ABSTRACT

In childhood B-cell precursor acute lymphoblastic leukemia, cytogenetics is important in diagnosis and as an indicator of response to therapy, thus playing a key role in risk stratification of patients for treatment. Little is known of the relationship between different cytogenetic subtypes in B-cell precursor acute lymphoblastic leukemia and the recently reported copy number abnormalities affecting significant leukemia associated genes. In a consecutive series of 1427 childhood B-cell precursor acute lymphoblastic leukemia patients, we have determined the incidence and type of copy number abnormalities using multiplex ligation-dependent probe amplification. We have shown strong links between certain deletions and cytogenetic subtypes, including the novel association between *RB1* deletions and intrachromosomal amplification of chromosome 21. In this study, we characterized the different copy number abnormalities and show heterogeneity of *PAX5* and *IKZF1* deletions and the recurrent nature of *RB1* deletions. Whole gene losses are often indicative of larger deletions, visible by conventional cytogenetics. An increased number of copy number abnormalities is associated with NCI high risk, specifically deletions of *IKZF1* and *CDKN2A/B*, which occur more frequently among these patients. *IKZF1* deletions and rearrangements of *CRLF2* among patients with undefined karyotypes may point to the poor risk *BCR-ABL1*-like group. In conclusion, this study has demonstrated in a large representative cohort of children with B-cell precursor acute lymphoblastic leukemia that the pattern of copy number abnormalities is highly variable according to the primary genetic abnormality.

Introduction

The cytogenetics of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is well documented, with specific chromosomal abnormalities used in risk stratification of patients for treatment.[1,2] Genomic studies have shown that copy number abnormalities (CNA) of genes involved in B-lymphocyte development and differentiation, cell cycle control and those of significance in hematopoiesis are common in BCP-ALL.[3-5] Notable deletions include *PAX5, IKZF1* (*Ikaros*),[6-11] and genes within the pseudoautosomal region (PAR1) of the sex chromosomes, resulting in the *P2RY8-CRLF2* gene fusion and overexpression of *CRLF2*.[12] Here there is particular interest in *IKZF1* and *CRLF2* in relation to outcome and their role as molecular targets for therapy. *IKZF1* deletions have been associated with a poor prognosis in BCP-ALL,[6-10] while the risk relating to *CRLF2* has been variable and dependent on other features.[13-15] Nevertheless, thus far these diverse findings have not led to any treatment changes. Studies have focused on small or selected cohorts and analyses have often been carried out independently from other genetic changes. Thus their accurate incidence, relationship to each other, and the major cytogenetic subgroups still have to be determined in order to understand their true clinical relevance.

Recently, we demonstrated that multiplex ligation-dependent probe amplification (MLPA) provided an accurate and reliable high throughput method to screen for CNA of the significant genes in BCP-ALL.[16] In this study, we screened a cohort of 1427 childhood BCP-ALL patients from two consecutive treatment trials using the same MLPA approach. We report the frequency and type of CNA involving these genes, their associations with established chromosomal abnormalities, and other clinical features.

Design and Methods

Patients in this study were diagnosed with BCP-ALL and registered on UK treatment trials UKALL97/99 (April 1997-June 2002) for children aged 1-18 years[17] and UKALL2003 (October 2003-July 2011) for children aged 1-25 years.[18] Clinical details were provided by the Clinical Trial Service Unit (CTSU), Oxford, UK. All participating centers obtained local ethical committee approval and written informed consent from patients, parents or guardians in accordance with the Declaration of Helsinki. Risk was assessed using National Cancer Institution (NCI) criteria.

Patients were classified into eight cytogenetic subgroups according to the presence of the following chromosomal abnormalities: 1) t(12;21)(p13;q22)/*ETV6-RUNX1* fusion; 2) high hyperdiploidy (51-65

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chromosomes); 3) translocations involving 11q23/MLL rearrangements; 4) t(9;22)(q34;q11)/*BCR-ABL1* fusion; 5) intrachromosomal amplification of chromosome 21 (iAMP21);[19] 6) t(1;19)(q23;p13)/*TCF3-PBX1*; 7) other abnormal (absence of abnormalities in subgroups 1-7 above); and 8) normal karyotype. Patients were classified into good, intermediate, and poor cytogenetic risk groups according to previously published data.[20]

DNA obtained from the presentation bone marrow sample was used to determine the copy number of *IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1*, and genes within PAR1: *CRLF2, CSF2RA, IL3RA*, using the SALSA MLPA kit P335 *IKZF1* (MRC Holland, The Netherlands), as previously described.[16] In those patients entered on UKALL2003, deletions of *IKZF1* and *RB1* were confirmed and further characterized by the P202 *IKZF1* and the P047 *RB1* SALSA MLPA kits, respectively.

Deletions of genes within the PAR1 region identified by MLPA were confirmed as *P2RY8-CRLF2* or unbalanced *IGH@-CRLF2* translocations by interphase fluorescence *in situ* hybridization (FISH) as previously reported.[12]

Statistical analysis was carried out using Intercooled STATA v. 12.0 (StataCorp, USA), particularly Wilcoxon Rank Sum for non-parametric assays and χ^2 for comparison of categorical variables.

Results

CNA among the entire cohort

In total, 1427 patients were included in this study. There was no difference between these patients and other trial participants with respect to sex, age, central nervous system (CNS) disease or NCI risk group. Tested patients were more likely to have a white blood cell (WBC) count over 10 x 10^9/L reflecting the increased possibility of surplus available material (*Online Supplementary Table S1*).

Incidences of CNA for genes tested by MLPA are given in Table 1. Overall, 59% of patients showed an abnormality of at least one of these genes: 433 (30%) patients had one, 254 (18%) had two, 131 (9%) had three and 28 (2%) had four or more deletions. Overall, deletions of *CDKN2A/B* and *ETV6* were the most frequent, while *EBF1* deletions were rare. Patients classified as NCI high risk were significantly more likely to have a greater number of deletions compared to those classified as NCI standard risk ($P<0.001$) (Figure 1).

Table 1 shows the distribution of abnormalities in relation to demographic and clinical features. The cohort comprised 665 (46%) females and 762 (54%) males. There was no shift in the gender balance within each subgroup according to the defined CNA. The median age of the cohort was five years (range 1-23) with 24% of patients being 10 years or older. Patients with *IKZF1* and *CDKN2A/B* deletions were significantly older. The median age for *IKZF1* and *CDKN2A/B* deletions was seven years (P <0.0001) and six years (P <0.0001), respectively, with 39% and 33%, respectively, of deletions occurring in patients aged 10 years old or older. The incidence of these deletions increased with age (Table 1), a trend that continued into adulthood as shown by the incorporation of MLPA data from the UK adult ALL treatment trial, UKALLXII[21] (Figure 2). There was a peak in incidence of *ETV6* deletions in children aged 2-4 years (*data not shown*), explained by their strong association with *ETV6-RUNX1*, which has a peak incidence in this age group.[22] There was no significant change in incidence linked to age for dele-

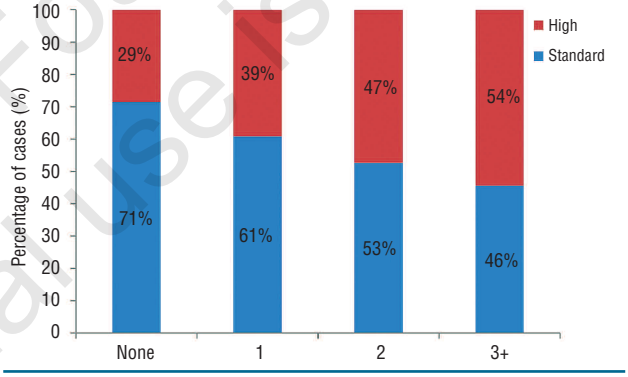

Figure 1. Bar chart showing the percentage of patients within NCI high and standard risk groups with increasing numbers of CNA.

Table 1. Association of copy number abnormalities with demographics and WCC.

	Cohort	Genes with CNA/chromosomal region																	
		IKZF1		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]	
N. of cases	1427	196	14%	272	19%	92	6%	395	28%	312	22%	30	2%	89	6%	63	4%
Age (years)																	
Median	5	7*		5		7		6*		4		6		6		5	
1-4	704	55	8%	135	19%	30	4%	152	22%	168	24%	10	1%	35	5%	29	4%
5-9	386	64	17%	66	17%	33	9%	115	30%	82	21%	14	4%	29	8%	26	7%
10-14	240	54	23%	53	22%	23	10%	93	39%	40	17%	6	3%	19	8%	5	2%
15-24	97	23	24%	18	19%	6	6%	35	36%	22	23%			6	7%	3	3%
Gender M:F (ratio)	762:665 (1.15)	105:91 (1.15)		146:126 (1.15)		50:42 (1.19)		214: 181 (1.18)		160:152 (1.05)		15:15 (1.0)		46:43 (1.07)		28:35 (0.8)	
WCC (x10^9/L)																	
Median	11.8	21.6*		21.3*		11.7		21.1*		13.1		13.9		14.8		22	
<10	633	65	10%	97	15%	36	6%	131	21%	130	21%	12	2%	37	6%	22	3%
10-49.9	527	69	13%	92	17%	42	8%	150	28%	118	22%	9	2%	32	6%	26	5%
≥50	267	62	23%	83	31%	14	5%	114	43%	64	24%	9	3%	20	7%	15	6%

CNA: copy number abnormalities; [1]*this group includes* P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations;* *P<*0.0001.*

tions of the other genes tested, including *ETV6* deletions in *ETV6-RUNX1* negative patients.

The median WBC count of the cohort was 11.8 x 10^9/L with 44% of patients having a count of less than 10 x 10^9/L. Patients with *IKZF1, PAX5* or *CDKN2A/B* deletions were more likely to have a WCC of over 50 x 10^9/L (each $P<0.001$) (Table 1). The association of these genes to age and WBC count meant that there was a significantly higher incidence of patients with *IKZF1, PAX5* and/or *CDKN2A/B* deletions classified as NCI high risk compared to other patients (each $P<0.001$) (Table 2).

The frequency of each cytogenetic subgroup among 1351 patients with a successful cytogenetic result is shown in Table 3. Patients with *ETV6-RUNX1*, high hyperdiploidy and those classified as 'other abnormal' comprised the most common subgroups at incidences of 28%, 30% and 24%, respectively. Patients positive for *ETV6-RUNX1* showed the highest number of CNA overall, followed by those in the other abnormal group. In contrast, CNA occurred at a lower than expected level in high hyperdiploid patients. The incidence of CNA was also low in the subgroup with MLL rearrangements and higher in the other poor-risk subgroups: *BCR-ABL1* positive and iAMP21. The increasing numbers of CNA in each cytogenetic group are shown in Figure 3, while the incidences and distribution of the individual CNA within each cytogenetic group are shown in Figure 4.

Among *ETV6-RUNX1* positive patients, in addition to a high incidence of wild-type *ETV6* deletions, *CDKN2A/B* and *PAX5* were each deleted in 22% of these patients, while the incidence of *IKZF1* deletions was low. Although *BTG1* deletions were rare throughout the cohort (n=87,

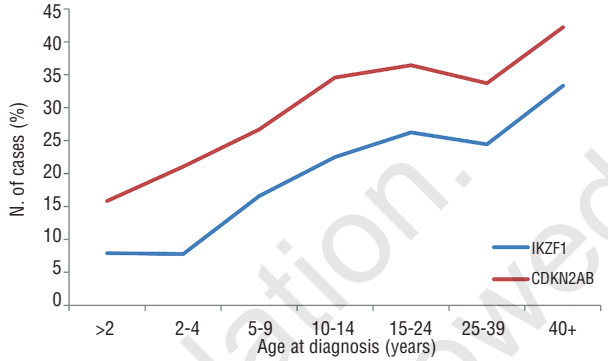

Figure 2. Graphs showing the proportion of cases with ***IKZF1*** and ***CDKN2A/B*** deletions according to age among ***BCR-ABL1*** negative patients. As it is known that there is a strong association between ***IKZF1*** deletions and ***BCR-ABL1*** positive ALL[7] and that the incidence of ***BCR-ABL1*** positive ALL increases with age[22], childhood ***BCR-ABL1*** positive patients were excluded from this age association analysis of ***IKZF1*** and ***CDKN2A/B*** for direct comparison with the MLPA tested adult ***BCR-ABL1*** negative series[21].

Table 2. Association of Copy Number Abnormalities with NCI and Cytogenetic Risk Group.

	Cohort		Genes with CNA															
	IKZF1		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]			
N. of cases	1351		177	13%	255	19%	89	7%	371	27%	301	22%	27	2%	87	6%	56	4%
NCI Risk Group																		
Standard	838	62%	71	8%	124	15%	53	6%	171	20%	191	23%	18	2%	51	6%	39	5%
High	513	38%	106	21%	131	26%	36	7%	200	39%	110	21%	9	2%	36	7%	17	3%
Cyto Risk Group																		
Good	787	58%	46	6%	101	13%	39	5%	153	19%	231	29%	14	2%	59	7%	10	1%
Intermediate	441	33%	96	22%	121	27%	23	5%	173	39%	57	13%	7	<1%	21	5%	32	7%
Poor	123	9%	35	28%	32	26%	27	22%	45	37%	13	11%	6	5%	7	6%	14	11%

Cyto, cytogenetics; [1]this group includes P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations.*

Table 3. Incidences of CNA according to cytogenetic subgroups.

Cytogenetic subgroup	Cases in each group		*IKZF1*		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]		Total CNA	
	N.	%[2]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[4]
Total	1351[5]		177	13%	255	19%	89	7%	371	27%	301	22%	27	2%	87	6%	56	4%	1334	
ETV6-RUNX1	379	28%	11*	3%	83	22%	29	8%	84	22%	203*	54%	14	4%	57*	15%	3*	<1%	484	6%
HeH	408	30%	35*	9%	18*	4%	10*	3%	65*	16%	28*	7%	0		2*	<1%	7	2%	165	12%
MLL rearranged	28	2%	2	8%	2	8%	0		8	31%	1	4%	0		0		0		13	1%
BCR-ABL1	33	2%	21*	64%	15*	45%	3	9%	16[5]	48%	1	3%	2	6%	1	3%	1	3%	60	5%
iAMP21	33	2%	7	21%	4	12%	13*	39%	4	12%	7	21%	3	9%	1	3%	10*	30%	49	4%
t(1;19)/*TCF3-PBX1*	44	3%	3	7%	8	18%	7	16%	10	23%	2	5%	1	2%	0		1	2%	32	2%
Other abnormal	324	24%	77*	24%	115*	36%	23	7%	153*	47%	51	16%	5	2%	3	1%	33*	10%	460	34%
Normal	102	8%	20	20%	9	9%	4	4%	22	22%	8*	8%	1	<1%	6	6%	1	1%	71	5%

[1]This group includes P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations; [2]Calculated from total cohort with a successful cytogenetic result; [3]Calculated as total number of patients in this specific cytogenetic subgroup with this CNA; [4]Calculated from total number of abnormalities, which is greater than the total number of patients; [5]Patients with a successful cytogenetics result only; *P<0.0001; [5]P=0.005.*

6%), they were frequently associated with *ETV6-RUNX1* (15%) ($P<0.0001$). There was an association between *BCR-ABL1* and *IKZF1* with 64% having a deletion of *IKZF1*. Deletions of *PAX5* and *CDKN2A/B* were also high in this subgroup at incidences of 45% and 48%, respectively.

Among iAMP21 patients, *RB1* deletions and *P2RY8-CRLF2* were observed at incidences (39% and 30%, respectively) significantly higher than expected ($P<0.0001$). They showed the second highest frequency of *ETV6* deletions. In the *TCF3-PBX1* subgroup, there was a high incidence of deletions of *PAX5* and *CDKN2A/B*. In the group classified as 'other abnormal', deletions of *CDKN2A/B*, *PAX5* and *IKZF1*, as well as *P2RY8-CRLF2*, were seen at higher than expected frequencies.

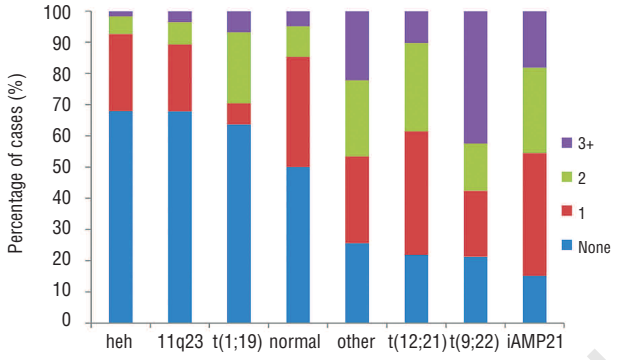

Figure 3. Bar chart showing the proportion of patients within each cytogenetic subgroup with increasing numbers of CNA.

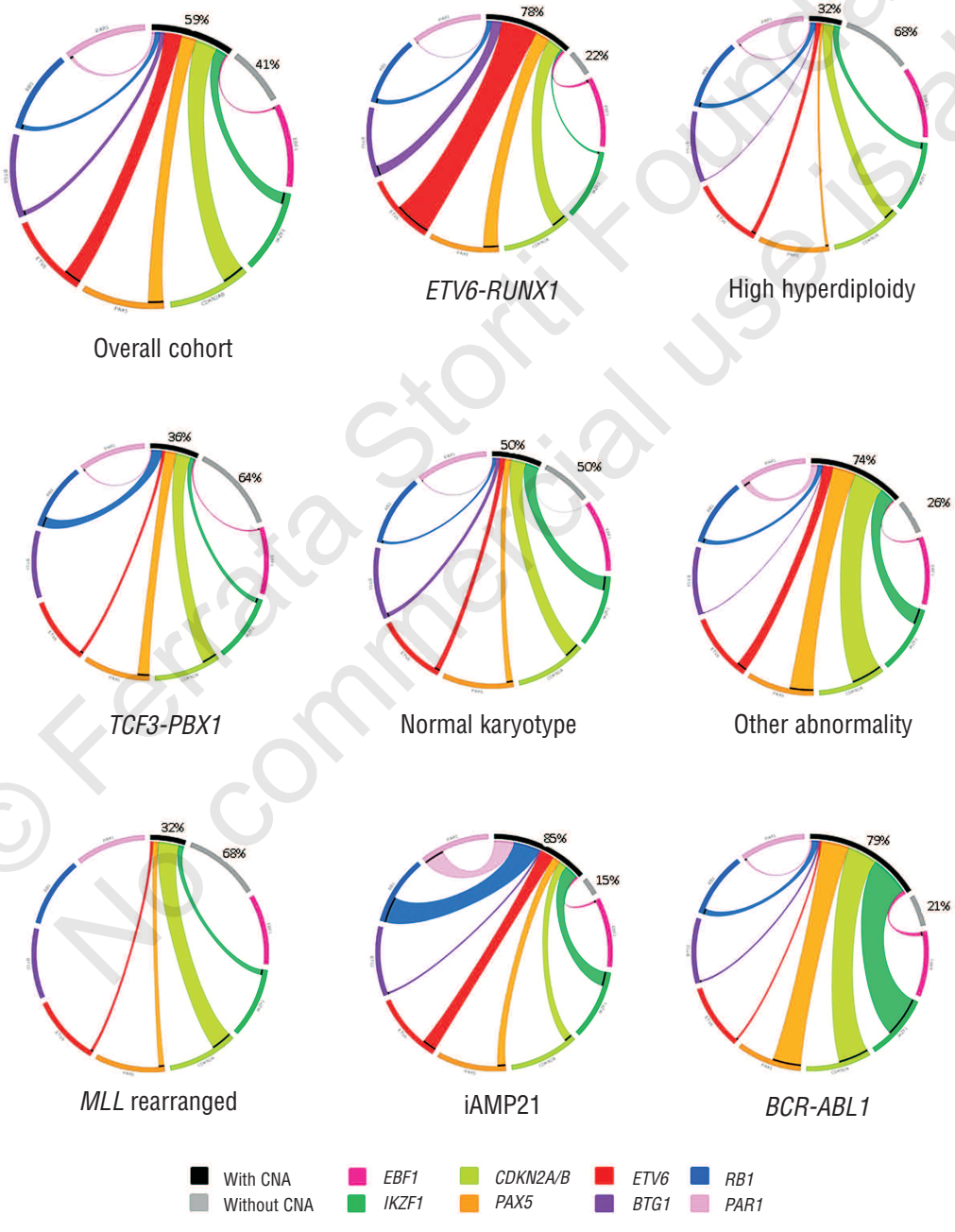

Figure 4. Circos plots showing the relative incidence and distribution of CNA within the overall cohort and the individual cytogenetic groups. The proportions of cases with and without CNA are indicated in black and gray, respectively, around the perimeter and the ribbons representing the individual CNA are color coded. The width of the ribbons reflects the frequency of each CNA.

Individual abnormalities

A range of interesting observations were made in relation to the individual abnormalities. IKZF1: deletions of *IKZF1* were present in 14% (n=196) of the cohort. The size of the deletion varied between patients (*Online Supplementary Figure S1*). The most frequent deletions involved either the whole gene (n=60) or were restricted to exons 4-7 (n=61). Other deletions occurred at lower frequencies: exons 2-3 (n=15), exons 2-7 (n=20), exons 4-8 (n=13), and miscellaneous deletions (n=27). Five patients had biallelic deletions; each showed a different pattern of loss, ranging from biallelic deletion of all exons to a subset of exons. No significant association was seen between the pattern of exon loss and cytogenetic subgroup, age or WBC count. Although unlikely to be significant, the biallelic deletions did not occur in association with any of the major cytogenetic groups. Whole gene deletions were associated with visible cytogenetic abnormalities of the short arm of chromosome 7 in 36 of 60 cases including: monosomy 7 (n=13), i(7)(q10) (n=7), dicentric chromosomes (dic) (n=6), balanced and unbalanced translocations involving chromosome 7 (n=10).

PAX5 and *CDKN2A/B*: a total of 272 (19%) patients showed heterogeneous CNA of *PAX5*. The majority of patients had deletions including exon 1 involving the entire or part of the gene (n=141). The remaining patients had partial deletions excluding exon 1 (n=121) or intragenic amplifications of exons 2 or 5 (n=10), as previously illustrated.[16]

Deletions of *CDKN2A/B* represented the most frequent abnormality in the cohort (n=395), of which 143 showed visible cytogenetic abnormalities of the short arm of chromosome 9 (9p), 212 showed no 9p abnormality, while 40 failed cytogenetic analysis. Among those cases with a visible 9p abnormality, 103 showed concurrent CNA of *PAX5*. A further 59 patients with CNA of both genes had no visible 9p abnormality (n=41) or failed cytogenetic results (n=18). The types of cytogenetic abnormalities involving chromosome 9 associated with these CNA are shown in *Online Supplementary Table S2*. Dicentric chromosomes involving chromosome 9 were shown in 43 patients: dic(7;9) (n=7), dic(9;12) (n=16) and dic(9;20) (n=20). Interestingly, they showed heterogeneous exon loss from PAX5, ranging from the entire gene to loss of the telomeric exons (not including exon 1) and they variably included deletions of *CDKN2A/B*.

ETV6: *ETV6* was frequently deleted throughout the entire cohort (n=312). As expected, *ETV6* deletions were frequent among *ETV6-RUNX1* positive patients (n=203). FISH results were available on 186 of these *ETV6-RUNX1* positive cases. In 154 (83%), the results by FISH and MLPA were concordant. Among the remaining 32 cases, deletions were found by MLPA but not FISH, indicating the presence of focal deletions below the resolution of FISH. Conversely, 40 cases with *ETV6-RUNX1* showed an *ETV6* deletion by FISH only. These either represented small populations of cells containing the deletions (<25% of nuclei) below the level at which MLPA would be expected to detect loss, or deletion of the wild-type *ETV6* allele with an associated gain of the derivative chromosome 21, thus producing a normal copy number for the *ETV6* exons covered by the MLPA probes. These observations highlight the previously described advantages and disadvantages of these two techniques.[16]

In total, 109 *ETV6-RUNX1* negative cases showed loss of *ETV6*; among 86 of these cases with FISH results available, 52 showed loss of the entire *ETV6* by both FISH and MLPA, while 34 cases showed small intragenic deletions by MLPA which were below the resolution of FISH.

RB1: deletions of *RB1* were present in 92 cases. These were of two types: 1) loss of the entire gene (n=60); 2) focal deletions, including exons 19-26 (n=28) (probes for these exons are included in the MLPA kit). An additional 4 cases had biallelic deletions of exons 19-26 as well as monoallelic loss of the remainder of the gene. Further studies using the P047-*RB1* MLPA kit on 65 cases confirmed whole gene loss in 40 cases tested. These studies indicated that the deletions extended into the adjacent genes: *ITM2B* and *RCBTB2* in all 40 cases, including *DLEU* in 38 of them. The relative location of these genes is shown in *Online Supplementary Figure S2A*. These results showed that *RB1* loss in these patients is part of a larger deletion targeting several genes.

Further characterization of those cases with focal deletions, using the *P047-RB1* MLPA kit with a higher probe density, showed the precise location of the deletion breakpoint to be between exons 17 and 18 in 19 of 21 cases tested, while in the 2 remaining cases the breakpoints were located between exons 16 and 17 and exons 18 and 19. In 8 of 21 cases with this focal deletion, the 5' breakpoint included deletion of the *RCBTB2* gene. The 4 patients with both deletions showed different patterns of exon loss: 2 showed monoallelic loss of *ITM2B, RCBTB2* and *DLEU*. The other 2 showed normal copy number for *ITM2B* and *DLEU* with the biallelic loss extending to *RCBTB2* in one case and monoallelic loss of this gene in the other. These results are illustrated in *Online Supplementary Figure S2B*.

From cytogenetic analysis, 52 cases in the entire cohort showed 13q abnormalities although only 52% (n=27) of these were associated with an *RB1* deletion.

CRLF2 *gene rearrangements*: deletions within PAR1 were detected by MLPA in 4% (n=63) of the cohort, of which 54 cases had fixed cells available for FISH investigations. The *P2RY8-CRLF2* fusion was confirmed by FISH in 49 cases, while the remaining 5 cases were found to be *IGH@-CRLF2* translocations with associated deletion within the PAR1 region. FISH testing for the presence of *IGH@* rearrangements had been carried out on 57% (n=807) of the entire cohort. Twelve cases, with a normal result by MLPA, were shown to have balanced *IGH@-CRLF2* translocations by FISH. Collectively, these patients were described as *CRLF2* rearranged, accounting for 5.3% of the cohort.

P2RY8-CRLF2 was most common among the iAMP21 subgroup (30%) and patients classified as 'other abnormal' (10%). Although *P2RY8-CRLF2* was rare in association with other cytogenetic subtypes, it was found in all subtypes in this series except the MLL rearranged group. The positive cases included 3 *ETV6-RUNX1* positive patients and one each of *TCF3-PBX1* and *BCR-ABL1*.

Among these patients with *CRLF2* rearrangements, 48% also showed loss of *IKZF1*. However, *IKZF1* deletions were more significantly associated with *IGH@-CRLF2* than *P2RY8-CRLF2*, occurring in 82% and 37%, respectively (P=0.001). *IGH@-CRLF2* patients were more likely to be classified as NCI high risk than *P2RY8-CRLF2* (65% and 29%, respectively, P=0.008). However, this result did not translate into a correlation between *IKZF1* deletion status and risk among *CRLF2* rearranged patients,

as 56% of high risk and 42% of standard risk *CRLF2* rearranged patients also had *IKZF1* deletions (*P*=0.22).

Discussion

In this study, we present the findings from a detailed retrospective analysis of CNA in significant genes involved in B-cell development, cell cycle control and hematopoiesis among a large consecutive series of pediatric BCP-ALL patients treated on UK ALL treatment trials. Although 59% of patients showed an abnormality of at least one of these genes, 41% showed none. The number of CNA occurring simultaneously in the same patient was low. Thus, these observations, in association with cytogenetic data, confirm that the genomic profiles of childhood BCP-ALL are not generally complex. Although the involvement of other genes not covered by the MLPA kit cannot be ruled out, data from SNP arrays have shown the incidence of other recurrent sub-microscopic abnormalities to be infrequent.[3-5] From MLPA studies, it is not possible to gain information on the temporal order in which these events arose in terms of karyotypic evolution or identify which abnormalities were the potential 'drivers' of leukemogenesis. However, it was possible to examine associations between these abnormalities and demographic and clinical features, as well as with cytogenetics.

In relation to the individual abnormalities, *IKZF1* showed heterogeneity in the size of the deletion, as previously demonstrated.[3] The majority of patients either showed deletion of the entire gene, often seen as a visible cytogenetic change involving 7p, or restricted to exons 4-7. A range of other deletion types were also observed of which a small number were biallelic. No significant association was found between the pattern of exon loss and cytogenetic subtype. *IKZF1* is transcribed in several isoforms as a result of alternative splicing, essentially altering the expression of exons 3 to 5 that encode the N-terminal DNA-binding domain. Deletions of exons 4-7 result in expression of a dominant negative IKZF1 isoform, Ik6, that lacks the N-terminal DNA binding zinc finger and shows oncogenic activity.[11,23] Deletion of exon 2, which harbors the translational start site, will inhibit protein translation. Loss of exon 8 will have an effect on dimerization of *IKZF1*. Thus, deletions involving these exons are likely to have the same impact as whole gene deletions. Loss of the non-coding exon 1 only is likely to be of no significance. Accurate characterization of these heterogeneous deletions of *IKZF1* is important before we can begin to understand their prognostic relevance.

The extent of *PAX5* deletions was variable, ranging from whole gene loss to loss of the telomeric exons, confirming previous SNP data[3] and our earlier observations based on FISH.[24] Those with deletions of the entire or part of the gene including exon 1 are predicted to result in reduced PAX5 expression. Those with partial deletions not involving exon 1 are predicted to express a mutant allele.[25] Ten patients showed intragenic amplifications of exons 2 or 5. These amplifications have been previously reported as a rare occurrence and are similarly predicted to express mutant alleles.[25] In a number of cases, *PAX5* deletions occurred as the result of dicentric chromosomes involving chromosome 9. Interestingly, among those cases with dic(9;12), 4 were associated with *ETV6-RUNX1* fusion, while the remaining 5 were classified as 'other abnormal'. Although the number of cases was small, there was a distinct pattern of exon loss between the two groups of dic(9;12) cases. The other abnormal cases showed loss of *ETV6* exons 1-2 and *PAX5* exons 5-10. This finding was consistent with previously published data in which this abnormality was associated with expression of an *ETV6-PAX5* fusion protein.[26] The dicentric chromosomes associated with *ETV6-RUNX1* showed larger deletions of ETV6 and loss of the entire *PAX5*, indicating that these dic(9;12) translocations do not result in *ETV6-PAX5* fusion.

Deletions of *CDKN2A/B* represented the most frequent abnormality in the cohort, which were often associated with visible abnormalities of 9p and concurrent loss of *PAX5*. Interestingly, 8 of the 10 cases showing intragenic amplification of *PAX5* also showed deletion of *CDKN2A/B*.

RB1 deletions were homogeneous compared to other deletions, being restricted to two types: 1) those including the entire gene, as well as the adjacent genes: *ITM2B, RCBTB2* and *DLEU*; and 2) focal deletions including exons 18-26 in all but one case. They were occasionally biallelic with different sized deletions on the two alleles. Some of the larger deletions were visible at the cytogenetic level. Deletions of 13q have been associated with increased risk of relapse.[20] However, this study showed that only approximately 50% of visible 13q abnormalities were associated with an *RB1* deletion, indicating that in at least some of these cases *RB1* is not the target of the deletion. RB1 deletions of exons 18-27 have been previously reported in lymphoma.[27] The molecular consequence of this recurring deletion is still not well understood. Expression of a truncated protein with altered function or deletion of *LPAR6/P2RY5*, located within *RB1*, may be one of the consequences.

With the exception of patients with *MLL* rearrangements, the presence of *CRLF2-P2RY8* has now been reported in association with all cytogenetic subtypes. In this study, we identified a small number of cases among patients with *ETV6-RUNX1*, *TCF3-PBX1* and *BCR-ABL1*; the latter two have not been previously reported. We showed *CRLF2* rearrangements to be present in 5.3% of the cohort. Several groups have reported a strong association between CRLF2 overexpression and *IKZF1* alterations.[13,14,28-30] Although this study is restricted to the detection of *CRLF2* rearrangements, with the exception of rare mutations of the gene,[31] without measure of expression, we confirmed this association. We also confirmed that *IGH@-CRLF2* occurred at a higher incidence in NCI high-risk patients, while *P2RY8-CRLF2* was seen at a higher frequency in NCI standard risk.

We have previously reported the high incidence of *P2RY8-CRLF2* among iAMP21 patients.[32] We confirmed an increased incidence of *ETV6* deletions as previously reported;[33] however, the frequent occurrence of *RB1* deletions in these patients is shown here for the first time.

There was some correlation between the distribution of CNA and patient age. Patients with *IKZF1* and *CDKN2A/B* deletions were older and their incidence increased with age. Patients with *IKZF1*, *PAX5* and *CDKN2A/B* deletions had significantly higher WBC count than patients with the other CNA. These associations with older age and higher WBC count explain why these deletions occur at a higher frequency in the NCI high-risk group, that is defined by age and WBC count. Previous studies have been inconclusive as to the prognostic relevance of *CDKN2A/B* in both childhood and adult ALL.[34]

The association with NCI risk defined here, coupled with observations that patients with *CDKN2A/B* deletions have a shorter time to relapse than other relapsed patients,[35,36] suggests that further studies are warranted in order to clarify the prognostic relevance of *CDKN2A/B* deletions.

The frequency of each cytogenetic subgroup among this patient cohort was the same as that previously reported by us for a single UK childhood ALL treatment trial, ALL97/99.[15,20] We showed that the incidence of CNA varied according to cytogenetic subtype, although the number did not correlate with the cytogenetic risk. For example, the good-risk cytogenetic groups (*ETV6-RUNX1* and high hyperdiploidy) as well as the poor risk (*BCR-ABL1* and *MLL* rearranged) showed a high and low number of CNA, respectively.

The high number of CNA in the *ETV6-RUNX1* positive group suggests that CNA rather than point mutations may be the drivers of leukemia in this subgroup, as other studies have shown *ETV6-RUNX1* positive leukemia to harbor a modest number of point mutations.[37] Interestingly, although *BTG1* deletions were rare, they were most often associated with *ETV6-RUNX1*, as previously reported.[38] As *BTG1* has been reported to be associated with glucocortoid receptor autoinduction,[39] these patients require follow up to determine whether these deletions affect their overall survival.

The lower than expected level of deletions in high hyperdiploid patients is unlikely to be an artefact of analyzing CNA in the context of ploidy change, as neither the genes tested nor the reference probes in the MLPA kit are located on chromosomes commonly gained in high hyperdiploidy. Point mutations have been found at an increased level in this subgroup, indicating that disease progression in high hyperdiploid patients may be driven by mutations rather than deletions, at least among the genes tested.[40] Although the incidence of CNA was also low in patients in the poor-risk subgroup with *MLL* rearrangements, this finding might be expected from the known potency of this abnormality as a driver of leukemogenesis.[41] From these observations, it is evident that future studies assessing the prognostic value of these CNA must include cytogenetic data in order to gain a clear picture of their association with outcome.

As previously shown, there was a strong association between *BCR-ABL1* fusion and *IKZF1* deletions.[7] Deletions of *PAX5* and *CDKN2A/B* were also high in the *BCR-ABL1* positive group. In the group classified as 'other abnormal', deletions of *CDKN2A/B*, *PAX5* and *IKZF1* were also frequent. The striking similarity in CNA profiles between these two groups is clearly shown in Figure 4. The poor-risk group described as *BCR-ABL1*-like,[6,41] defined as sharing the same gene expression profile as well as the same poor risk as *BCR-ABL1* positive patients, is most likely to be found among those patients with 'other abnormal' karyotypes as no distinctive karyotypic features have yet been described to define them. Thus the occurrence of deletions of *CDKN2A/B*, *PAX5* and *IKZF1*, as well as deregulated *CRLF2*, among patients in this 'other abnormal' group may provide a pointer to the BCR-ABL1-like subgroup, as indicated by others.[42]

This study represents the largest trial-based screen for abnormalities in selected genes of significance in the development of BCP-ALL. It has confirmed findings of previous studies of associations between copy number abnormalities and particular cytogenetic subgroups. It has shown the heterogeneous nature of deletions such as *PAX5* and *IKZF1* and the recurrent nature of *RB1* deletions. The association of *IKZF1* and *CDKN2A/B* deletions with NCI high risk is of interest, and within the 'other abnormal' cytogenetic group, the presence of these deletions and/or rearrangements of *CRLF2* may point to the poor-risk *BCR-ABL1* like group.

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