EJC Paediatric Oncology 3 (2024) 100140

Contents lists available at ScienceDirect

EJC Paediatric Oncology

journal homepage: www.journals.elsevier.com/ejc-paediatric-oncology

Original research

Targeted treatment options for paediatric B-cell precursor acute lymphoblastic leukaemia patients with constitutional or somatic chromosome 21 alterations

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ARTICLE INFO

Keywords:
Acute lymphoblastic leukemia
RAS
JAK
Paediatric
Chromosome 21
Down syndrome
High hyperdiploid
ETV6::RUNX1
IAMP21

ABSTRACT

Background: Chromosome 21 is affected in ~60% of paediatric B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) patients and includes somatic and constitutional gains, intrachromosomal amplification of chromosome 21 (iAMP21), and the translocation t(12;21) resulting in the *ETV6::RUNX1* gene fusion.
Methods: Since these numeric and structural chromosome 21 alterations are not targetable, we studied the type and frequency of yet-proven targetable events co-occurring with chromosome 21 alterations.
Results: Among 307 primary paediatric BCP-ALL cases, JAK/STAT pathway lesions were most frequent in patients with constitutional gain of chromosome 21 (Down syndrome ALL; 35/71, 49%) and iAMP21 (9/22, 41%). RAS pathway lesions were most frequent in high hyperdiploidy (62/108, 57%) and *FLT3* lesions were most frequent in iAMP21 (7/22, 32%). Virtually all cases expressed CD19 and CD22 at the cell surface. Positivity for CD20 surface expression ranged from 67% in iAMP21 (8/12) to 20% in *ETV6::RUNX1* (26/129).
Conclusion: Activated JAK/STAT, RAS or FLT3 signalling, and CD marker surface expression may provide targetable treatment options for the majority of chromosome 21-altered BCP-ALL cases.

1. Introduction

Paediatric B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) is characterized by different initiating lesions including gene fusions and aneuploidy. [1] These subtypes often involve chromosome 21 and have varying impacts on prognosis, indicating the importance of chromosome 21 alterations. Children with Down syndrome (DS), characterized by a constitutional gained chromosome 21, have a higher risk of developing ALL, and are at a higher risk of relapse. [2,3] Intrachromosomal amplification of chromosome 21 (iAMP21) is generally characterized by a poor prognosis, although the outcome greatly improves when treated with higher intensity chemotherapy schedules. [4] In the good prognostic high hyperdiploid subtype (HeH; 51–65 chromosomes), chromosome 21 is the most frequently gained chromosome; 96% of cases gain at least one copy of chromosome 21, while 76% of cases gain at least two copies. [5] The good prognostic *ETV6::RUNX1* subtype arises from a translocation between chromosome 12 and chromosome 21 and often has additional gains in chromosome

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https://doi.org/10.1016/j.ejcped.2023.100140
Received 16 October 2023; Received in revised form 19 December 2023; Accepted 20 December 2023
Available online 23 December 2023

21. [6] A gained copy of chromosome 21 can also be found in other BCP-ALL subtypes. [7].

Despite ongoing improvements in the outcome of paediatric patients with BCP-ALL, relapse still occurs in 8% of cases in the DCOG ALL-11 study. [8] Children with a bone marrow relapse occurring later than 6 months after stopping therapy have survival rates over 80%. [9] However, the prognosis of high-risk relapsed BCP-ALL patients remains poor, with a 10-year event-free survival of only 19–24% and an overall survival of only 20–38%. [10] These relapsed patients are thus insufficiently rescued with chemotherapy and hematopoietic stem cell transplantation, illustrating the need for more targeted treatment directed to biological features of these therapy-resistant leukemic cells. In addition, targeted treatment might reduce treatment-related toxicity, also in patients with a relatively favourable prognosis. Studies in DS BCP-ALL indicate that a trisomy of chromosome 21 is associated with presence of specific targetable lesions, such as an activated JAK-pathway due to a rearranged *CRLF2* chemokine receptor together with *JAK2* mutation and an activated MEK-ERK pathway due to mutations in *KRAS*. [11,12] For paediatric BCP-ALL patients with somatic numeric or structural chromosome 21 alterations, the frequency of these targetable events has not been characterized in detail. In the present study, we analysed the type and frequency of targetable lesions and expressed CD markers in the context of chromosome 21 aberrations, which may serve as a roadmap to develop relevant targeted trials for these patients.

2. Patients and methods

2.1. Patients

Initial diagnosis samples of paediatric BCP-ALL patients from the following study groups were included: Dutch Childhood Oncology Group (DCOG ALL-8, ALL-9, ALL-10, and ALL-11), German Cooperative ALL (COALL 06–97 and 07–03), UKALL (UKALL2003 and UKALL2011; only DS), and ANZCHOG (ANZCHOG ALL8 and AIEOP-BFM ALL 2009; only DS). In accordance with the declaration of Helsinki, written informed consent to use excess diagnostic material for research purposes was obtained from parents or guardians, as approved by the medical research ethics committee for each collaborative group. *ETV6*::*RUNX1*, *BCR*::*ABL1*, *TCF3*::*PBX1*, iAMP21, *KMT2A* rearrangement and ploidy status were determined using fluorescence in situ hybridization (FISH), karyotyping, copy number array and/or RT-PCR by country or study group diagnostic reference laboratories. Chromosome 21 gains were called based on karyotype or DNA arrays. A patient was classified as having a gain of chromosome 21 material if one of the techniques showed gain of the entire or part of chromosome 21. Subclonal gains of chromosome 21 were also classified as a chromosome 21 gain. We excluded near haploid and low hypodiploid cases (<40 chromosomes) and cases with >65 chromosomes. In addition, we excluded *BCR*::*ABL1* patients, as these are already treated with tyrosine kinase inhibitors since 2005. Immunophenotyping was performed by flow cytometric reference laboratories and positivity for a marker was defined as at least 10% positive cells.

2.2. RNA sequencing

RNA was isolated from mononuclear cells using TRIzol. Paired-end total RNA sequencing was performed as described before [13] at Novogene Co., Ltd. on the NovaSeq 6000 platform (> 50 million raw reads). Reads were aligned to the GRCh38.p12 human genome reference. Read counts per gene were calculated using STAR v2.6.0c. Normalization of library size was done with TMM using EdgeR v3.32.1. Fragments per kilobase per million (FPKM) was calculated and batch correction was performed using ComBat (sva v3.38.0). t-SNE plots were created with the Euclidean distance based on Spearman correlations using expressed genes (counts per million ≥1 in two samples) as input. Fusion detection was done using STAR-Fusion v1.4.0 and FusionCatcher v1.00. Variants present in more than 0.1% of the general population, as annotated by dbSNP release 155, were excluded. Variant calling was done using GATK v4.1.2.0. Variants with a variant allele frequency (VAF) below 10% or with 15 or less total reads at the variant position were excluded. We focused on variants in specific genes and positions (Table S1) that were classified as pathogenic or likely pathogenic in ClinVar.

2.3. Analysis

All analyses were performed with R version 3.6.3. The following packages were used: ggplot2 version 3.3.2, circlize version 0.4.12, and ComplexHeatmap version 2.6.2.

3. Results

3.1. Patient cohort

We studied the frequency of targetable lesions and CD marker expression in chromosome 21-altered groups in a retrospective BCP-ALL cohort enriched for chromosome 21 alterations (Figure S1). Of the 582 patients, 307 were available for RNA sequencing (clinical characteristics in Table S2) to determine targetable genetic lesions (71 DS-ALL, 22 iAMP21, 108 HeH, 47 *ETV6*::*RUNX1*, 15 somatic chromosome 21 alteration, and 44 no chromosome 21 alteration; Table S3). DS-ALL was defined as a separate group as treatment decisions are often dictated by the presence of DS. Within our 71 DS patients, there were 9 *ETV6*::*RUNX1* patients, 7 HeH patients, and 2 iAMP21 patients. To evaluate CD marker expression, we used flow cytometry data from 378 patients (35 DS-ALL, 12 iAMP21, 111 HeH, 130 *ETV6*::*RUNX1*, 16 somatic chromosome 21 alteration, and 74 no chromosome 21 alteration).

3.2. Gene expression profiling

We evaluated the effect of chromosome 21 aberrations on overall gene expression. Clustering based on the expression of all genes was driven by the leukemic subtype; especially *ETV6*::*RUNX1* patients clustered closely together, and to a lesser extend HeH patients (Fig. 1A). Chromosome 21 aberration was secondary to the primary genetic subtype regarding gene expression clustering, as DS-ALL with *ETV6*::*RUNX1* clustered together with non-Down syndrome *ETV6*::*RUNX1* patients. Similarly, *ETV6*::*RUNX1* patients with a somatic chromosome 21 gain clustered with other *ETV6*::*RUNX1* patients (Fig. 1A and B). iAMP21 patients did not show a strong gene expression profile and clustered together with the heterogeneous B-other group in our cohort. In general, patients with a chromosome 21 gain, regardless constitutional or somatic, showed higher expression of chromosome 21-located genes (Fig. 1C). iAMP21 patients, followed by DS-ALL and HeH patients, tended to have the highest expression of certain chromosome 21-located genes, whereas B-other and *ETV6*::*RUNX1* patients without a chromosome 21 gain tended to have lower expression of chromosome 21-located genes.

3.3. Frequency of targetable lesions in the context of chromosome 21 alterations

In total, 191 mutations or small insertions or deletions (indels, including internal tandem duplications [ITDs]) were detected, of which 69% (131/191) clonal (≥25% VAF) and 31% (60/191) subclonal (<25% VAF) (Table S4). Furthermore, 59 gene rearrangements were detected (Table S5). JAK/STAT pathway lesions were most frequent in DS-ALL (49%) and iAMP21 (41%; Table 1), including *CRLF2* rearrangements associated with increased *CRLF2* expression (Figure S2). DS-ALL patients with a JAK/STAT lesion all had a *CRLF2* rearrangement (34/35) or an activating *CRLF2* mutation (1/35; Fig. 2). In 15 out of the 34 DS-ALL

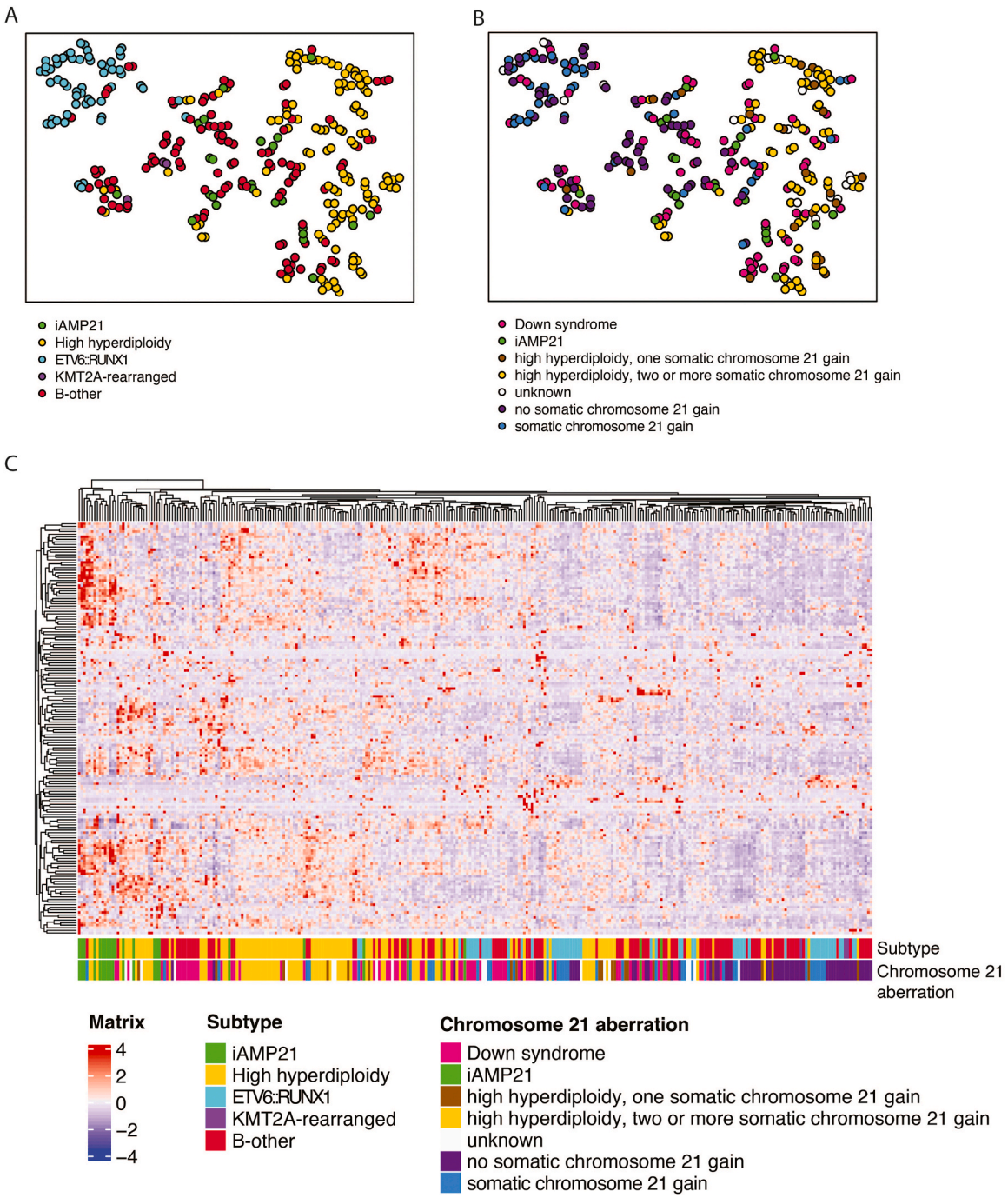

Fig. 1. Gene expression clustering of the RNA sequencing cohort. t-SNE plot based on all expressed genes with each dot representing a patient, coloured for A) subtype and B) chromosome 21 aberration. C) Heatmap showing scaled expression of all chromosome 21-located (expressed) genes, using complete linkage clustering. Rows represent genes and columns represent patients. Abbreviations: iAMP21, intrachromosomal amplification of chromosome 21.

cases with a *CRLF2* rearrangement, an additional mutation in *JAK1* or *JAK2* (44%) was found. *CRLF2* lesions were also frequent in iAMP21 (27%, 6/22), samples with chromosome 21 gain (33%, 5/15) and samples without chromosome 21 gain (18%, 8/44). These were accompanied by a *JAK1* or *JAK2* mutation in 50% (3/6), 60% (3/5), and 63% (5/8) of the cases, respectively. The JAK/STAT pathway lesions affecting *EPOR* and *IL7R* occurred in less than 10% of samples. Although low in number, all JAK/STAT pathway lesions observed in HeH occurred in patients who gained two or more copies of chromosome 21 and in *ETV6*::*RUNX1* patients with a somatic gain of chromosome 21.

Mutations in the RAS pathway genes *NRAS* and *KRAS* were found at similar frequencies within each subgroup (Table 1; Fig. 2) and were most frequent in HeH (26% and 31%, respectively). Clonal *NRAS* and *KRAS* mutations were always mutually exclusive, whereas a subclonal mutation in one gene was found to co-occur with a clonal or subclonal mutation in the other gene in six HeH patients.

FLT3 lesions were most frequent in iAMP21 (32%; Table 1; Fig. 2). In addition to missense mutations, ITDs and in-frame insertions in *FLT3* were identified. *FLT3* RNA expression was comparable or higher than *FLT3* RNA expression in *KMT2A*-rearranged samples in 14% of DS-ALL, 23% of iAMP21, 16% of HeH, 0% of *ETV6*::*RUNX1*, 13% of somatic chromosome 21 alteration, and 5% of non-*KMT2A*-rearranged samples without a chromosome 21 alteration (Figure S3).

HeH samples were divided into samples with one gained copy of

Table 1
Frequency of targetable lesions related to chromosome 21 status.

	DS-ALL	iAMP21	HeH	*ETV6*: *RUNX1*	Somatic chromosome 21 alteration[a]	No chromosome 21 alteration[a]
Total of patients	71	22	108	47	15	44
JAK/STAT signalling	35 (49%)	9 (41%)	3 (3%)	2 (4%)	5 (33%)	10 (23%)
CRLF2	35 (49%)	6 (27%)	2 (2%)	2 (4%)	5 (33%)	8 (18%)
fusion	34	6	2	2	5	8
clonal	1	0	0	0	1	2
subclonal	1	0	0	0	0	0
JAK2	11 (15%)	2 (9%)	0	0	3 (20%)	6 (14%)
fusion	0	0			0	1
clonal	9	2			2	5
subclonal	2	0			1	1
JAK1	4 (6%)	1 (5%)	1 (1%)	0	0	1 (2%)
clonal	2	1	1			1
subclonal	2	0	0			0
EPOR	0	1 (5%)	0	0	0	1 (2%)
fusion		0				1
clonal		1				0
subclonal		0				0
IL7R	1 (1%)	2 (9%)	0	0	0	0
clonal	1	2				
subclonal	0	0				
RAS signalling	16 (23%)	4 (18%)	62 (57%)	8 (17%)	2 (13%)	9 (20%)
NRAS	8 (11%)	1 (5%)	28 (26%)	5 (11%)	0	6 (14%)
clonal	8	0	18	4		3
subclonal	1	1	11	1		3
KRAS	7 (10%)	2 (9%)	33 (31%)	2 (4%)	1 (7%)	2 (5%)
clonal	5	1	21	0	1	1
subclonal	2	1	12	2	0	1
PTPN11	2 (3%)	1 (5%)	10 (9%)	1 (2%)	1 (7%)	2 (5%)
clonal	1	0	7	1	1	1
subclonal	1	1	3	0	0	1
FLT3	7 (10%)	7 (32%)	14 (13%)	0	2 (13%)	5 (11%)
clonal	6	5	11		2	3
subclonal	1	2	4		0	2
Any targetable lesion	50 (70%)	17 (77%)	73 (68%)	10 (21%)	9 (60%)	21 (48%)

[a] For included subtypes, refer to Table S3
The number of fusions, clonal and subclonal mutations can add up to more than the total in case multiple mutations exist in the same sample.

chromosome 21 (21/95, 22%) and two or more gained copies of chromosome 21 (74/95, 78%), excluding 13 patients without specified chromosome 21 status. Out of the targetable lesions, only *KRAS* was more frequently mutated in HeH cases with one gained copy of chromosome 21 (57%, 12/21) compared with those having two or more gained copies of chromosome 21 (26%, 19/74; $p = 0.009$; Table S6). As there were very few lesions observed in *ETV6*::*RUNX1* samples, cases with and without (partial) gain of chromosome 21 were not compared.

Lesions in RAS, JAK/STAT, and FLT3 pathways were mutually exclusive in 89% (160/180) of the cases with any targetable lesion (Fig. 3). Lesions in the JAK/STAT and RAS pathways co-occurred in 5.6% (10/180), lesions in the JAK/STAT and FLT3 pathways co-occurred in 2.8% (5/180), and lesions in the RAS and FLT3 pathways co-occurred in 2.8% (5/180). The ratio of clonal:subclonal lesions did not significantly differ per pathway or per chromosome 21 alteration subtype (Fisher test p-values >0.05 for all comparisons; data not shown). In summary, the highest frequency of targetable genetic lesions in JAK/STAT, RAS, and FLT3 pathways was found in iAMP21 (77%), DS-ALL (70%), HeH (68%), and other subtypes with chromosome 21 gain (60%; Table 1). Targetable lesions were identified in a substantial group of patients with high minimal residual disease levels ($\geq$0.01%) at end of induction: 42% showed a RAS pathway aberration, 14% a JAK/STAT pathway aberration and 10% a *FLT3* aberration (Table S7).

3.3.1. Frequency of CD marker positivity in the context of chromosome 21 alterations

We determined the frequency of CD19, CD20, and CD22 positivity ($\geq$10% positive cells) in chromosome 21-altered subtypes. All but two patients were positive for CD19 and all, but four patients were positive for CD22 (data not shown). CD20 positivity was found in 46% of DS-ALL, 67% of iAMP21, 39% of HeH, 20% of *ETV6*::*RUNX1*, 44% of patients with a somatic chromosome 21 alteration, and 48% of patients without a chromosome 21 alteration (Fig. 4). High CD20 expression, defined as expression in $>$ 50% of cells, was found in 14% of DS-ALL, 33% of iAMP21, 15% of HeH, 7% of *ETV6*::*RUNX1*, 0% of patients with a somatic chromosome 21 alteration, and 27% of patients without a chromosome 21 alteration (Fig. 4).

4. Discussion

We evaluated the frequency of potential targets for targeted drugs and immunotherapies in newly diagnosed paediatric BCP-ALL patients with chromosome 21 alterations. Chromosome 21 aberrations did not strongly dictate gene expression profiles but did affect expression of chromosome 21-located genes. We found at least one targetable genetic lesion affecting the RAS, JAK/STAT or FLT3 pathway in 77% of iAMP21, 70% of DS-ALL, 68% in HeH, 21% in *ETV6*::*RUNX1*, 60% in other cases with a chromosome 21 gain, and 48% of BCP-ALL cases without a chromosome 21 alteration. Almost all patients were positive for CD19 and CD22, and 39% for CD20 surface expression. Clinical outcome analysis was not meaningful given the relatively small patient subgroups and the different treatment protocols administered to this compiled group of patients. Previous studies showed that clonal mutations in RAS-family genes, but not subclonal mutations, are associated with an unfavourable outcome in BCP-ALL children treated on contemporary treatment protocols. [14] In addition, clonal *KRAS* mutations are frequent at relapse and associated with a poor prognosis in these cases. [15] *JAK2* aberrations are associated with a higher incidence of relapse compared with *JAK2* wildtype HeH, *ETV6*::*RUNX1*, and *TCF3*::*PBX1* cases, but not B-other/*BCR*::*ABL1*-like [16], while *CRLF2* rearrangements are associated with a higher incidence of relapse in the COG [17], but not in the DCOG and CoALL study groups [18]. *FLT3*-ITD, but not

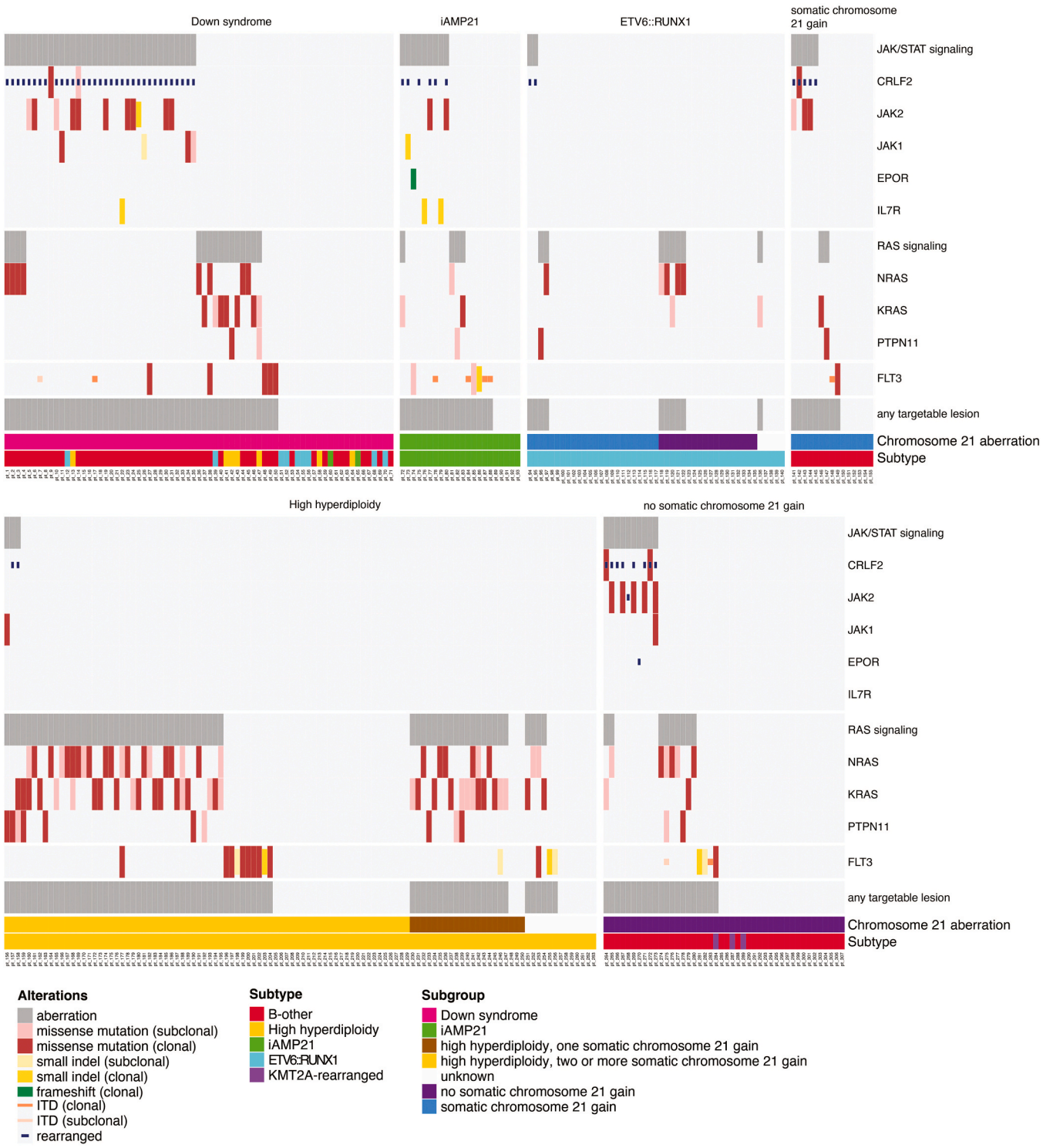

Fig. 2. Frequency of actionable events in the context of chromosome 21 alterations. Lesions were determined using total RNA sequencing and patients were divided based on genetic subtype and chromosome 21 alteration. Each row represents a gene, each column represents a patient. Abbreviations: chr21, chromosome 21; iAMP21, intrachromosomal amplification of chromosome 21; ITD, internal tandem duplication; indel, small insertion or deletion.

FLT3 mutations, are associated with a poor prognosis in paediatric acute myeloid leukaemia [19], but to our knowledge, their effect on prognosis has not been studied in paediatric ALL. *FLT3* overexpression confers a poor prognosis in infant *KMT2A*-rearranged ALL. [20].

In correspondence with other studies, we found the highest frequency of RAS pathway mutations in HeH (57%) and the highest frequency of *CRLF2* rearrangements in DS-ALL (49%). [12,14,16–18] We here show more specifically that the frequency of *KRAS* mutations is higher in HeH cases with one gained copy compared with those who gained two or more copies of chromosome 21. *KRAS* mutations may functionally cooperate with genes located on chromosome 21 resulting in the deregulation of cell division and B-cell differentiation. [11] We previously noticed that a small group of RAS-mutated HeH cases had a poor outcome in the DCOG ALL-10 study. [14] At present, it is unknown whether this poor prognostic group represents *KRAS*-mutated HeH cases with an additional copy of chromosome 21.

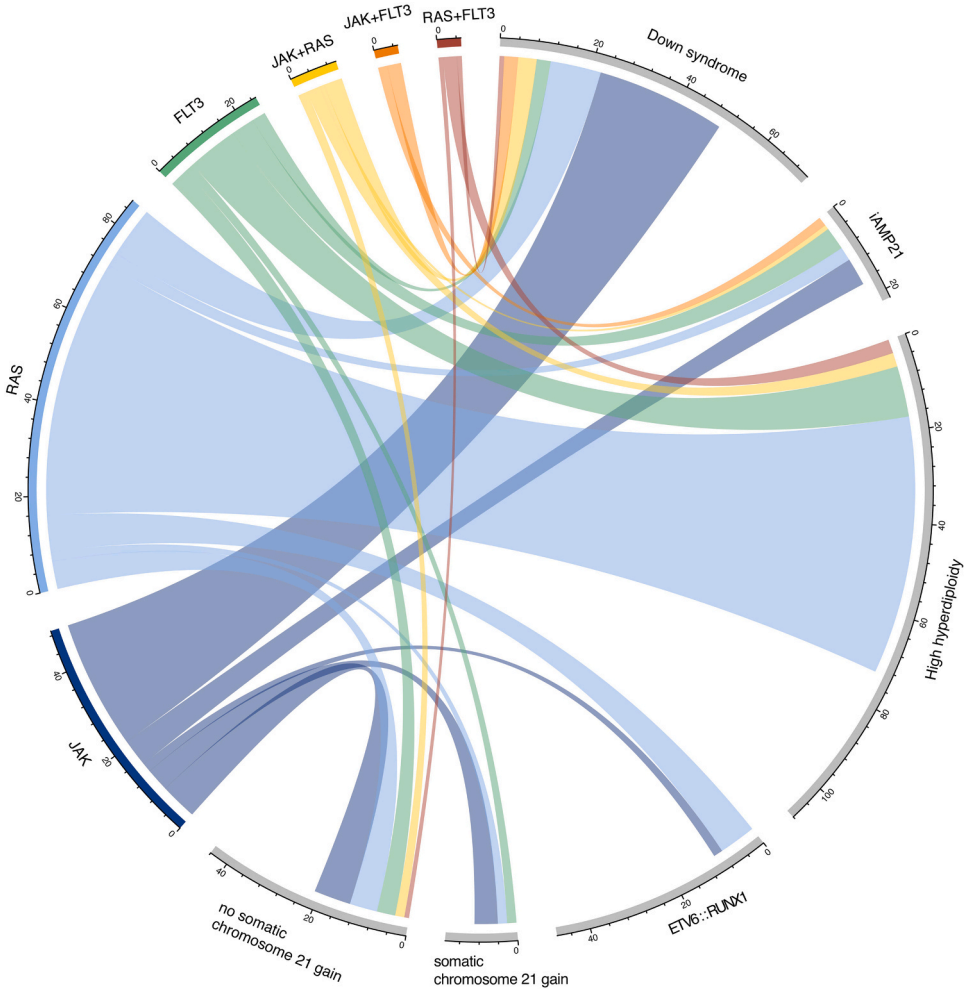

Fig. 3. Circosplot showing the frequency of RAS, JAK/STAT and/or FLT3 pathway activation per subtype. Summarized data of Fig. 2 presented in a circosplot showing the overlap and mutually exclusiveness of the identified activated pathways. Ribbons connect the pathways with the subtypes, colors indicate the type of lesion. Abbreviations: iAMP21, intrachromosomal amplification of chromosome 21.

DS-ALL forms a heterogeneous subgroup within BCP-ALL with both targetable and more recently identified lesions. [21] Implementing targeted drugs and replacing conventional chemotherapeutic drugs in upfront treatment protocols may be especially beneficial for DS-ALL patients given their high vulnerability for treatment-related toxicity. [2,22] RAS pathway inhibition was successful in RAS-mutated ex vivo samples [11,14,15,23] and in vivo xenograft studies. [11,15] Inhibition of MEK, downstream of RAS, synergized with glucocorticoids in the induction of cell death in BCP-ALL cells. [14,24,25] This observation led to the SeluDex trial, combining selumetinib with dexamethasone, for relapsed/refractory ALL. [26] In the present study, with newly diagnosed cases, we observed that RAS pathway mutations were more frequent than JAK/STAT pathway alterations in HeH, indicating that MEK/ERK inhibitors may be the preferred choice for more upfront inclusion of targeted treatment for this subtype. JAK/STAT pathway inhibition is promising in ex vivo studies [16], and in vivo xenograft studies. [27,28] A clinical trial evaluating ruxolitinib efficacy in diagnostic BCP-ALL patients with a JAK/STAT lesion is ongoing (NCT02723994) and a case-study of two patients indicates activity of ruxolitinib against *EPOR*-rearranged BCP-ALL. [29] We here showed that in patients with constitutional (i.e. DS-ALL) or somatic gain of chromosome 21 (non-HeH), as well as in iAMP21 patients, the JAK/-STAT pathway alterations are more frequent than RAS pathway alterations. This may therefore favour JAK/STAT inhibitors as first choice if both type of inhibitors have equal efficacy and specificity.

FLT3 overexpression is common in infant *KMT2A*-rearranged ALL. [30] Single-agent midostaurin in a phase I/II study in these patients showed limited efficacy and will therefore be evaluated in combination with chemotherapy in acute myeloid leukaemia patients. [31] A randomized study with lestaurtinib in *KMT2A*-rearranged infant ALL showed clinical benefit for patients who achieved potent FLT3 inhibition and/or had ex vivo sensitivity to the FLT3 inhibitor. [32] In addition, an ongoing clinical trial evaluates the addition of gilteritinib to chemotherapy for *FLT3*-ITD paediatric acute myeloid leukaemia patients. FLT3 inhibitors may also be beneficial for high-risk subtypes such as iAMP21, that currently require intensive treatment, [4,33] since we here show that one third of these cases have FTL3-ITD or kinase domain mutations. In all studied subgroups, except for *ETV6::RUNX1*, some cases showed high *FLT3* expression, to a level comparable to *KMT2A*-rearranged cases. These individual patients might also benefit from FLT3 inhibition to a similar degree as the *KMT2A*-rearranged patients.

Recently, the immune-directed therapies inotuzumab ozogamicin [34,35], an anti-CD22 antibody conjugated to calicheamicin, blinatumomab [36], a bispecific T-cell engager (BiTE) targeting CD19, and chimeric antigen receptor T-cell (CAR-T) [37] targeting CD19, were shown to be effective for relapsed and refractory BCP-ALL patients. Precision medicines against targetable lesions could provide a bridging option towards CAR-T treatment or provide an alternative in case of immunotherapy failure. Given the higher frequency of CD20 positivity in the high-risk iAMP21 subtype, rituximab [38,39] or the new

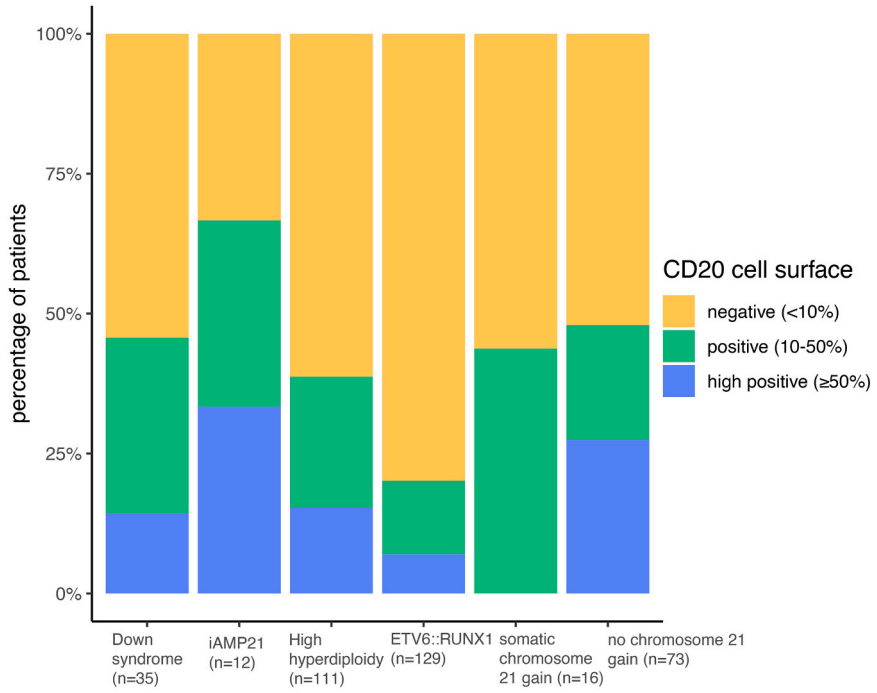

Fig. 4. Frequency of CD20 marker positivity in the context of chromosome 21 alterations. CD20 marker cell surface expression in the chromosome 21-altered subtypes is shown. Positivity is defined by at least 10% of cells positive for CD20, high positivity is defined by at least 50% of cells positive for CD20. Abbreviations: iAMP21, intrachromosomal amplification of chromosome 21.

anti-CD20 BiTE glofitamab [40] might be valuable treatment options for iAMP21 patients. The ultimate choice for a CD marker directed therapy not only depends on the presence of the CD marker, but also on the level of expression (fluorescence intensity), percentage of positive blasts, and the stability of the marker expression at the cell surface. [34].

In conclusion, the frequency of yet-proven targetable genetic lesions (21–77%) and CD marker expression (99%) is high in chromosome 21-altered BCP-ALL, pointing to a large group of paediatric patients for which targeted drugs may be beneficial.

Funding

This work was supported by the Foundation Pediatric Oncology Center Rotterdam (SKOCR), the Princess Máxima Center Foundation, the Dutch Cancer Society (grant KWF-10482), the KiKa Foundation (Kika-264 grant), Oncode Institute, and core funding to Prof. Dr. Monique L. den Boer.

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Sonneveld Edwin: Resources, Writing – review & editing. **Boeree Aurélie:** Investigation, Writing – review & editing. **Hormann Femke M.:** Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Boer Judith M.:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Michels Naomi:** Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **den Boer Monique L.:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Zwaan C. Michel:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Pieters Rob:** Resources, Writing – review & editing. **Beverloo H. Berna:** Resources, Writing – review & editing. **Sutton Rosemary:** Resources, Writing – review & editing. **Escherich Gabriele:** Resources, Writing – review & editing. **Moorman Anthony V.:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interest.

Data availability

Data can be shared upon request.

Acknowledgements

We thank the participating study groups for collecting and providing genetic and clinical information. Primary childhood leukaemia samples used in this study were provided by the Blood Cancer UK Childhood Leukaemia Cell Bank, the Sydney Children's Tumor Bank Network, and the biobanks of the Dutch Childhood Oncology group and the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia. We thank Alex Q. Hoogkamer for his contribution to the computational analyses.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcped.2023.100140.

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