Multiclonal complexity of pediatric acute lymphoblastic leukemia and the prognostic relevance of subclonal mutations

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ABSTRACT

Genomic studies of pediatric acute lymphoblastic leukemia (ALL) have shown remarkable heterogeneity in initial diagnosis, with multiple (sub)clones harboring lesions in relapse-associated genes. However, the clinical relevance of these subclonal alterations remains unclear. We assessed the clinical relevance and prognostic value of subclonal alterations in the relapse-associated genes IKZF1, CREBBP, KRAS, NRAS, PTPN11, TP53, NT5C2, and WHSC1 in 503 ALL cases. Using molecular inversion probe sequencing and breakpoint-spanning polymerase chain reaction analysis we reliably detected alterations with an allele frequency below 1%. We identified 660 genomic alterations in 285 diagnostic samples of which 495 (75%) were subclonal. RAS pathway mutations were common, particularly in minor subclones, and comparisons between RAS hotspot mutations revealed differences in their capacity to drive clonal expansion in ALL. We did not find an association of subclonal alterations with unfavorable outcome. Particularly for IKZF1, an established prognostic marker in ALL, all clonal but none of the subclonal alterations were preserved at relapse. We conclude that, for the genes tested, there is no basis to consider subclonal alterations detected at diagnosis for risk group stratification of ALL treatment.

Introduction

Improvements in the treatment of pediatric acute lymphoblastic leukemia (ALL) have resulted in high overall survival rates, now approaching 90%.1,2 Nevertheless, relapse still remains the most common cause of treatment failure and death in children with ALL, and better recognition of individuals at risk of developing relapse will likely aid further improvements in outcome. Recent studies describing the genomic landscape of relapsed ALL have shown that relapse often originates from a minor (sub)clone at diagnosis, at a cellular fraction often undetectable by routine diagnostic methods.3-5 These minor (sub)clones harbor genomic alterations acquired later during leukemia development, which could potentially contribute to clonal drift, but are unlikely to be essential for initiation of the primary disease. However, selective pressure of the upfront treatment may provide a competitive advantage to subclones that harbor alterations in cancer genes, enabling their selective survival, eventually leading to treatment failure. Both the number and clonal burden of the alterations in these genes are expected to be increased at the time of relapse, compared to initial diagnosis. Indeed, mutations in relapse-associated genes, such as those in the histone acetyltransferase (HAT) domain of the histone methyltransferase CREBBP, can often be traced back to minor subclones in the diagnostic sample.4,6,7
Genomic characterization of relapsed pediatric ALL has revealed multiple alterations that are enriched compared to diagnosis, including activating mutations in RAS pathway genes, HAT domain mutations in CREBBP and deletions or mutations in the B-cell transcription factor IKZF1. The presence of these aberrations at the time of diagnosis can be of potential prognostic relevance, as has been demonstrated extensively for IKZF1 in many different treatment protocols and can even lead to adjustments in stratification and treatment. However, it remains unclear whether mutations in relapse-associated genes when present in a minor subclone at initial diagnosis are also clinically relevant.

Subclonal mutations can be identified using deep targeted, next-generation sequencing techniques. Despite the sensitivity of these techniques, both amplification and sequencing can easily lead to errors that hamper the reliable detection of low-level mosaic mutations. We previously demonstrated that single molecule molecular inversion probes (smMIP), which use unique molecular identifiers to barcode each DNA copy, can correct for sequencing and amplification artefacts, resulting in a reliable detection of low-level mosaic mutations, down to a variant allele frequency of 0.4%.

In this study we used the smMIP-based sequencing approach to perform deep targeted sequencing of seven relapse-associated genes in a cohort of 508 pediatric ALL samples taken at initial diagnosis, resulting in the detection of 141 clonal and 469 subclonal mutations. In addition, we performed real time quantitative polymerase chain reaction (PCR) to sensitively detect subclonal IKZF1 exon 4-7 deletions (del 4-7), which were found at a similar frequency of these mutations ranged from 0.03-100%.

Results

A total of 503 diagnostic samples from children with ALL (Online Supplementary Table S1) was subjected to targeted deep sequencing of the relapse-associated genes TP53, CREBBP (HAT domain), KRAS, NRAS, PTPN11, NT5C2 and WHSC1 using smMIP, which contain random molecular tags to accurately detect low-level mosaic variants. Each targeted region was covered with an average of 309 unique capture-based consensus reads (Figure 1), enabling the reliable detection of alterations with allele frequencies even below 1%. A total of 7,836 quality-filtered variants was detected, of which 610 were absent in public and private variant databases and were predicted as pathogenic. The allele frequency of these mutations ranged from 0.03-100% (Figure 2A, Online Supplementary Table S3). The majority of the mutations (473/610; 78%) was found in one of the three RAS pathway genes (KRAS, NRAS, PTPN11), of which 418 (88%) were known hotspot mutations.

In addition to sequencing the seven relapse-associated genes, we performed sensitive screening for IKZF1 deletions, which are strongly associated with the occurrence of relapse. We chose to focus on exon 4-7 deletions, which represent 25% of all IKZF1 deletions, have a similar unfavorable outcome as other IKZF1 deletions, and show the strongest clustering of deletion breakpoints, thus enabling their sensitive upfront detection by breakpoint-spanning semi-quantitative PCR. Applying this strategy to the 503 diagnostic samples revealed all 22 IKZF1 exon 4-7 deletions.
tions previously identified using a standard MLPA method, as well as 28 additional cases carrying deletions that were missed with the MLPA technique. All breakpoints were sequenced to determine their unique break-point-spanning sequences (Online Supplementary Table S6). Using a dilution series of a control sample with a full-clonal IKZF1 exon 4-7 deletion, we determined the level of clonality of the deletions, which ranged from 100% down to 0.32% (Figures 1 and 2, Online Supplementary Figure S1C). All but one of the subclonal IKZF1 exon 4-7 deletions had allele frequencies below 10% (Online Supplementary Table S7).

Subclonal alterations in relapse-associated genes are common at diagnosis

Combining sequence mutations and IKZF1 exon 4-7 deletions, we detected 660 genomic alterations in 285 diagnostic samples, of which 165 (25%) were present in the major fraction of cells (allele frequency ≥25%), which were referred to as high-clonal. The remaining 495 mutations (75%), most of which had an allele frequency <10%, were referred to as subclonal (Online Supplementary Figure S2, Online Supplementary Table S7). A total of 147/285 patients carried at least one alteration in a major clone, while 138/285 (48%) patients carried exclusively subclonal alterations. NRAS and KRAS were the most frequently affected genes, showing major clone mutations in 6% and 8% of the cases and subclonal mutations in 20% and 15% of the cases, respectively (Figure 2A, B).

The proportion of subclonal alterations, relative to major clone alterations, was variable among different genes, ranging from 59% for IKZF1 exon 4-7 deletions to 86% for PTPN11 mutations (Figure 2B). Only one thus far unknown (subclonal) NT5C2 mutation (p.Arg507Trp) was identified in a leukemia sample from a patient who did not relapse (Figure 2A, B). Subclonal mutations were relatively common in hyperdiploid ALL (184 cases), particularly for mutations in RAS pathway genes (190/256; 74%), WHSC1 (22/26; 85%) and CREBBP (13/27; 48%) (Online Supplementary Table S8). Major clone WHSC1 mutations were mostly identified in ETV6-RUNX1-positive cases (4/10, 40%).

Potency of RAS pathway genes as drivers of clonal expansion

We identified 473 RAS pathway mutations in 225/503 (45%) cases, of which 78% were subclonal (median allele frequency = 3.5%). Over half of the RAS-affected cases were hyperdiploid (>47 chromosomes), in line with previous studies indicating that RAS mutations are associated with hyperdiploidy at diagnosis.10,33 The abundance of these mutations in major and minor clones suggests that these mutations drive clonal expansion during the development of leukemia. Major clone RAS pathway mutations (n=102; all being known hotspots) were found to be mutually exclusive, and 52/102 (51%) of these RAS-mutated cases had at least one additional subclonal mutation in one of the three RAS pathway genes. The mutations mostly affected codons 12 and 13 of KRAS and NRAS (Figure 3A-C), and considerable variability in the level of clonality was observed between the different RAS hotspot mutations at the time of diagnosis. For example, NRAS G12A (10 cases), NRAS G12V (7 cases), and PTPN11 E76K (7 cases) were never found to be present in a major clone, whereas 55% (n=11) of the KRAS G13D and 27% (n=9) of the KRAS G12D mutations were found in major clones. With these high numbers of RAS mutations, the variability in clonal burden between hotspot mutations may provide an opportunity to compare the capacity of different hotspots to drive clonal expansion of ALL. In order to test this hypothesis we compared allele frequencies and performed statistical analyses. We found that KRAS hotspot mutations had a significantly higher allele frequency compared to both NRAS and PTPN11 mutations (Wilcoxon signed-rank test, P<0.01) (Figure 3D). When comparing the different hotspot mutations within KRAS, A146V showed the lowest allele frequency, indicating a weaker potential of this hotspot to drive clonal expansion compared to the other KRAS hotspots. Furthermore, the allele frequency of KRAS G13D was significantly higher than...
Figure 2. Prevalence and distribution of alterations in eight relapse-associated genes. (A) Violin plot showing the variability in mutation allele frequency at diagnosis in the genes studied. The color of the dots indicates whether the mutation was detected in a case without relapse (blue) or with relapse (red). (B) Bar plot showing the frequencies of major clone (high-clonal) and subclonal alterations per case in the genes studied. RAS pathway genes (NRAS, KRAS, PTPN11) were the most frequently mutated. Subclonal mutations (yellow bar) were highly prevalent in all genes tested. A subset of cases had both clonal and subclonal alterations in the same gene (gray bar).
that of the NRAS hotspot mutations G13D, G12D and KRAS A146V (Figure 3E). This finding indicates that some RAS hotspot mutations (e.g., KRAS G12D, G13D, A146T) may result in a stronger expansion potential compared to others (e.g., NRAS A146V, NRAS G12D, G13D), and further illustrates the complex heterogeneity of RAS hotspot mutations in their potential to drive clonal expansion.

### Relevance of gene alterations to relapse development

The high number of alterations in these relapse-associated genes at the time of diagnosis triggers the hypothesis that these could be used as prognostic biomarkers for relapse development, even when present at subclonal levels. To test this hypothesis, we first explored whether alterations in each of the eight genes were enriched in diagnostic samples from patients who subsequently relapsed compared to diagnostic samples from patients who did not relapse. In general, subclonal alterations were very common at primary diagnosis in patients who relapsed (60/82; 73%) as well as in patients who did not relapse (165/203; 81%). For high-clonal alterations, we only observed a higher percentage of relapse development in cases with IKZF1 deletions compared to wild-type cases, whereas an association with relapse development was not observed for diagnostic samples with subclonal alterations in any of the genes, including IKZF1 (Figure 4).

Furthermore, patients with high-clonal IKZF1 4-7 deletions were more often classified as having high residual disease (MRD; >5x10^{-4} at day 79 or 84 after start of the treatment) in both representative ALL9 and ALL10 cohorts (Fisher exact test, P<0.01 and P<0.05, respectively), compared to patients without an IKZF1 deletion (Online Supplementary Table S10). The CIR at 5 years was 41.7% (SE 0.04%) and 42.9% (SE 0.03%) in patients with high-clonal IKZF1 4-7 deletions treated according to the ALL9 and ALL10 protocols, respectively (Figure 5). The cause-specific hazard ratio (HR_CS) in the two representative cohorts (n=376), estimated with a univariate Cox proportional hazards regression model, revealed an association of high-clonal IKZF1 exon 4-7 deletions with relapse (HR=7.22; 95% CI: 3.27-15.95; P<0.01). In the multivariate Cox model, in which age at diagnosis, gender and MRD status were included, the adjusted HR_CS was 3.6 (95% CI: 1.38-9.55; P<0.01) (Table 1, Online Supplementary Table S11). These data are in line with those from earlier studies on these cohorts in which all IKZF1 deletions were included. However, when we assessed the clinical relevance of subclonal alterations for relapse development in IKZF1, or any of the other genes, we did not find a significant association in the combined ALL9 and ALL10 cohorts compared to wild-type cases (Table 1, Online Supplementary Table S11), and the CIR was similar in the two groups (Figure 5). Furthermore, patients with subclonal IKZF1 4-7 deletions did not have significantly different levels of MRD compared to IKZF1 wild-type patients (Online Supplementary Table S10). Since previous studies have shown a lack of association of IKZF1 deletion with relapse in patients who carry a deletion in ERG, we used MLPA to test whether there was an enrichment of ERG deletions in cases with subclonal IKZF1 exon 4-7 deletions compared to those with clonal IKZF1 exon 4-7, but these deletions were infrequent in both groups (Online Supplementary Table S12).

### Tracing of major and minor clone mutations at the time of relapse

To obtain further insight into the clinical relevance of the identified alterations in relapse development, we investigated whether these were preserved in the cases that relapsed. For this analysis, we used all 146 cases that later developed a relapse, of which 82 carried alterations in a major or minor clone in one or more of the genes (Online Supplementary Tables S13 and S14). Overall, we found that for most genes at the time of diagnosis the frequency of subclonal alterations was similar or slightly higher compared to that of the alterations detected in a major clone (Online Supplementary Figure S3A, Online Supplementary Tables S13 and S14).

We collected 73 relapse samples from patients who carried these major or minor clone alterations at the time of diagnosis (39%), which enabled us to trace 171 of the 185 sequence mutations, and 25 of the IKZF1 exon 4-7 deletions. We did not assess whether mutations detected at diagnosis were still preserved in minor clones at relapse, since these clones were unlikely to be true relapse drivers. Overall, 56% (22/39) of the tested major clone mutations were found to be preserved in the major clone at relapse, whereas the value for the subclonal mutations was 7%
Figure 3. Potency of RAS pathway mutations to drive clonal expansion. (A-C) Schematic representation of KRAS (A), NRAS (B) and PTPN11 (C) indicating the prevalence of common hotspot mutations. (D) Violin plot showing allele frequency in hotspot mutations of three investigated RAS pathway genes. The median allele frequency was significantly higher in KRAS, indicating a high potential of KRAS hotspot mutations to drive clonal expansion. (E) Violin plot showing allele frequencies in frequent KRAS and NRAS hotspot mutations. The median allele frequency was significantly higher in KRAS G13D, suggesting their higher potential to drive clonal expansion compared to other RAS hotspot mutations.
For Ikzf1 exon 4-7 deletions, the difference was even more striking. Here, the presence of deletions was studied in 19 available relapse samples using breakpoint-spanning PCR, followed by Sanger sequencing to confirm that the breakpoint sequences were identical at diagnosis and relapse (Figure 6, Online Supplementary Figure S3B, Online Supplementary Tables S6 and S7). All major clone Ikzf1 exon 4-7 deletions were found to be preserved in the major clone at the time of relapse (n=12), which is in agreement with earlier findings and illustrates their relevance to relapse development in these treatment protocols. In contrast, none of the subclonal exon 4-7 deletions in Ikzf1 (n=15) was preserved in either the major or a minor clone at relapse. Collectively, the data from the present study indicate that these deletions, when present at initial diagnosis at a subclonal level, do not drive relapse in pediatric ALL.

Discussion

ALL is a heterogeneous disease in which specific genomic alterations show strong associations with relapse risk and outcome. In this study, we assessed the clinical relevance and prognostic value of subclonal alterations in eight genes frequently mutated in relapsed B-cell precursor ALL in a cohort of 505 diagnostic samples. Our data demonstrate that subclonal alterations in these genes are very common at the time of diagnosis, but that these mutations do not provide a basis for risk stratification in
Our smMIP analysis. If already present at the time of initial diagnosis, sampling for these subclonal deletions in diagnostic samples is still feasible, even though they are only obtained at detectable levels. The presence of IKZF1 deletions has been shown to be associated with relapse and survival in multiple clinical ALL studies, and these deletions have been described as playing a role in resistance to tyrosine kinase inhibitors and glucocorticoids. Therefore, with the advance of more sensitive detection techniques, the question of whether subclonal alterations are also associated with relapse is very relevant, both from biological and from clinical perspectives. We here demonstrate that, in contrast to major clone IKZF1 exon 4-7 deletions, cases that carry this deletion only in a subset of the cells do not show an association with relapse. Moreover, whereas all major clone exon 4-7 deletions were preserved in cases that relapsed, none of the relapses from cases with subclonal exon 4-7 deletions at diagnosis carried this deletion. Importantly, the majority of subclonal deletions had allele frequencies below 10% (Online Supplementary Table S7). Therefore, since a threshold to distinguish subclonal from major clonal deletions is difficult, deletions closer to our threshold of 25% should be evaluated with caution. Nevertheless, the difference between major and minor clone IKZF1 4-7 deletions is striking, and the reason behind this remains unclear. Possibly, the functional impact of full-clonal IKZF1 deletions, which arise early during leukemia development, is different from that of deletions that occur in later stages when the leukemia has already expanded. Other deletions in IKZF1 show much less clustering in their breakpoints and, therefore, screening for these subclonal deletions in diagnostic samples is much less efficient. We did not, therefore, directly assess the stability and potential prognostic importance of whole gene and rare intragenic IKZF1 deletions. However, a previous study showed that other IKZF1 deletion subtypes...
have similar prognostic relevance as exon 4-7 deletions, suggesting that subclonal alterations in these other IKZF1 deletions may show the same lack of association.

In summary, we show that subclonal alterations in the relapse-associated genes IKZF1, CREBBP, KRAS, NTRK3, PTPN11, TP53, and WHSC1 in pediatric ALL are frequently present at initial diagnosis, often at a subclonal level. At relapse, however, most of these subclonal mutations are lost, suggesting that their selective advantages over wild-type clones during treatment is limited. This finding has direct implications for clinical practice, particularly in the case of IKZF1, where deletion status is used for routine risk stratification. We conclude that, at least for the investigated set of genes, there is no basis for the use of subclonal alterations at initial diagnosis as a prognostic marker.

Disclosures
No conflicts of interest to disclose.

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Contributions
RPK, EW, Fvd., and AGvK conceived the study. RPK, EW, ŽA and JY designed the study. PMH and RP are responsible for the clinical outcome data of patients included in the study. ŽA, JY, Svr, Avd. LD and WHS performed experiments and analyzed the data. ŽA and MF performed statistical analyses. ŽA, JY and RPK wrote the manuscript and created the figures and tables. ES provided samples and clinical information. All authors critically reviewed the manuscript and approved the final submitted manuscript.

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