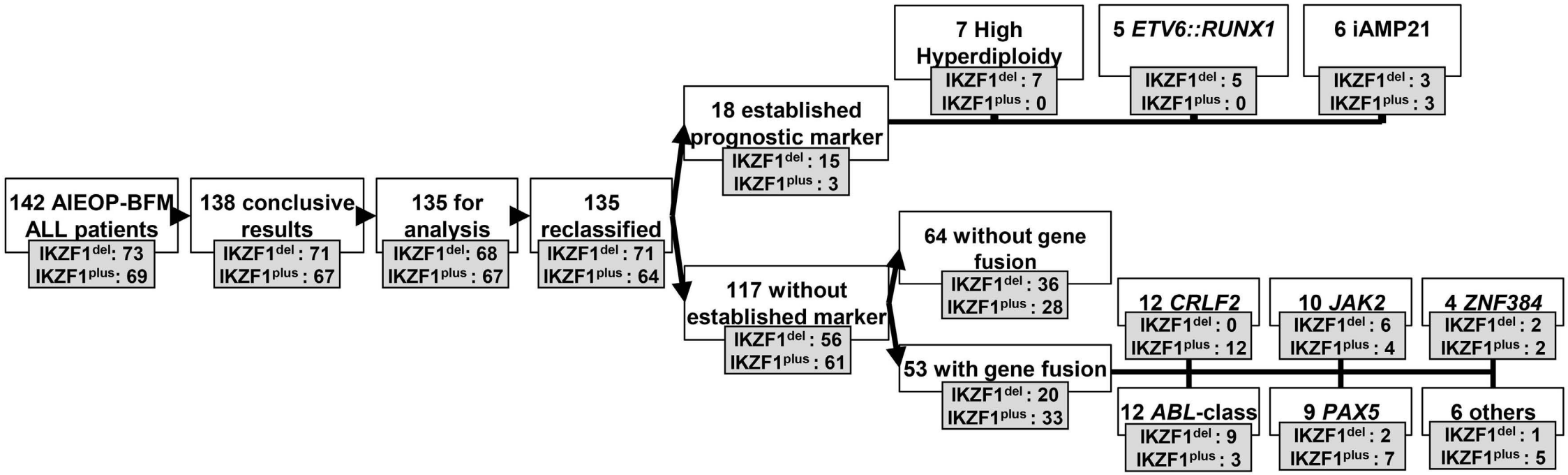
Deciphering the molecular complexity of the IKZF1^{plus} genomic profile using Optical Genome Mapping

Acute lymphoblastic leukemia (ALL) is the most frequent pediatric cancer. Genetic stratification is one of the hallmarks of the advancement of therapeutic intervention in pediatric B-cell precursor ALL (BCP-ALL) and led to an improved overall survival of >90%.[1-3] Despite these advancements, a proportion of patients still face challenges such as relapse or therapy-related toxicities.[2] Deletions targeting the lymphoid transcription factor *IKAROS Family Zinc Finger 1 (IKZF1)* occur in approximately 15% of pediatric B-ALL and have been associated with adverse outcome and as a predictor of relapse.[3-6] Moreover, when *IKZF1* deletion co-occurred with at least one additional deletion in *CDKN2A, CDKN2B* (homozygous deletion only), *PAX5*, or PAR1 (*P2RY8::CRLF2*) in the absence of ERG deletion, so-called IKZF1^{plus} patients showed a very poor prognosis in the AIEOP-BFM ALL 2000 trial.[7] The prognosis of IKZF1^{plus} was, however, dependent on measurable minimal residual disease (MRD), arguing for other leukemic drivers.[7] To systematically assess the underlying genetic complexity of *IKZF1*-deleted BCP-ALL, we performed a retrospective genome-wide analysis utilizing Optical Genome Mapping (OGM), enabling the detection of all kinds of numerical and structural variants (SV) in one approach. Ultra-high molecular weight DNA was extracted from frozen peripheral blood or bone marrow cells from pediatric *BCR::ABL1*-negative BCP-ALL patients with either *IKZF1* deletion (IKZF1^{del}) or IKZF1^{plus} profile within the AIEOP-BFM ALL 2000 and 2009 trials (*Online Supplementary Table S1*). We show here that half of the patients displayed additional favorable or unfavorable prognostic markers, including *ETV6::RUNX1*, high hyperdiploidy (HeH), iAMP21, and gene fusions. Of interest, *ETV6::RUNX1* and HeH were absent in IKZF1^{plus} patients. Importantly, when excluding patients positive for *ETV6::RUNX1*, HeH, or iAMP21, a similar event-free survival (EFS) was observed for both groups, questioning the prognostic relevance of the IKZF1^{plus} profile.

A total of 142 patients were included in this study and OGM (Bionano Genomics) was performed as previously described (Figure 1A).[8] Informed consent was obtained from all patients involved in the study or from their legal representatives. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Hannover Medical School Ethics Committee (N. 8657_BO_K_2019, 11/03/2020). Conclusive results were obtained from 138 patients which were 98.2% (1,171/1,193) concordant to pre-existing multiplex ligation-dependent probe amplification (MLPA) data with respect to *IKZF1, PAX5, CDKN2A, CDKN2B,* PAR1, *BTG1, EBF1, ETV6* and *RB1* copy number status. For the detection of *P2RY8::CRLF2*, originating from an ~300 kbp deletion in the subtelomeric PAR1 region on chromosome X and Y, which is not completely resolved in the hg19 reference, the reference genomes hg38 or CHM13 (T2T) were used for mapping, resulting in improved resolution (*Online Supplementary Figure S1*). According to our data, 3 patients had to be excluded from further analysis: one due to a t(9;22)(q34.12;q11.23) resulting in *BCR::ABL1* fusion, and 2 HeH patients for whom OGM did not confirm an *IKZF1* deletion. Furthermore, 5 patients were reclassified due to the identification of an additional small deletion in *PAX5* (exon 6-7), a previously undescribed intragenic *ERG* deletion, or because no deletion in *PAX5*, *CDKN2A/B*, or PAR1 could be detected. Overall, 94.9% (131/138) of the patients showed concordance regarding the IKZF1$^{del/plus}$ definition. The final cohort consisted of 135 patients, including 71 classified as IKZF1^{del} and 64 classified as IKZF1^{plus} (Figure 1A).

In 52.6% (71/135) of the BCP-ALL patients in this study, genetic alterations with (putative) prognostic impact were present in addition to IKZF1$^{del/plus}$ (Figure 1B). HeH, *ETV6::RUNX1*, or iAMP21 were detected in 18 patients. Patients with HeH and *ETV6::RUNX1* were exclusively found in the IKZF1^{del} group. In 39.3% (53/135) of the cases, an additional alteration leading to a gene fusion was detected and validated on transcript level (Figure 1B; *Online Supplementary Table S2*). Gene fusions were found in both subgroups and grouped into *ABL*-class, *CRLF2, JAK2, PAX5, ZNF384*, and other fusions (Figure 1A). The *CRLF2* group consisted only of *P2RY8::CRLF2* fusions, which by definition allocated these patients to the IKZF1^{plus} subgroup. Five patients with *PAX5::JAK2* fusions were allocated to the *JAK2* group. We detected simple and complex SV that led gene fusions on a transcript level (*Online Supplementary Table S1*); for example, a simple inv(1)(q24.2q25.2) (Figure 2A), a derivative chromosome 1 including three inversions (Figure 2B), and a complex chromosome 1 involving a duplication and insertion (Figure 2C), that all led to *RCSD1::ABL2* fusions. In the last case, an ~150 kbp segment containing exon 1-3 of *RSCD1* was inserted in inverted orientation in between an ~150 kbp duplicated segment containing *ABL2* exon 5-12. Similar to the findings by Stanulla *et al.*,[7] a dismal 5-year EFS for patients with IKZF1^{plus} was observed compared to IKZF1^{del} and IKZF1^{WT} (Figure 3A). The cumulative incidence of relapse (CIR) was increased in IKZF1^{plus} patients (Figure 3B). Interestingly, the differences in 5-year EFS of IKZF1^{plus} and IKZF1^{del} were no longer seen when HeH, *ETV6::RUNX1*, and iAMP21 were excluded from the analysis (0.61±0.06 *vs.* 0.70±0.06) (Figure 3C). This can be explained by the fact that no patient with HeH or *ETV6::RUNX1* experienced adverse events and these were found exclusively in the IKZF1^{del} group. However, IKZF1^{plus} patients were still more likely to

A



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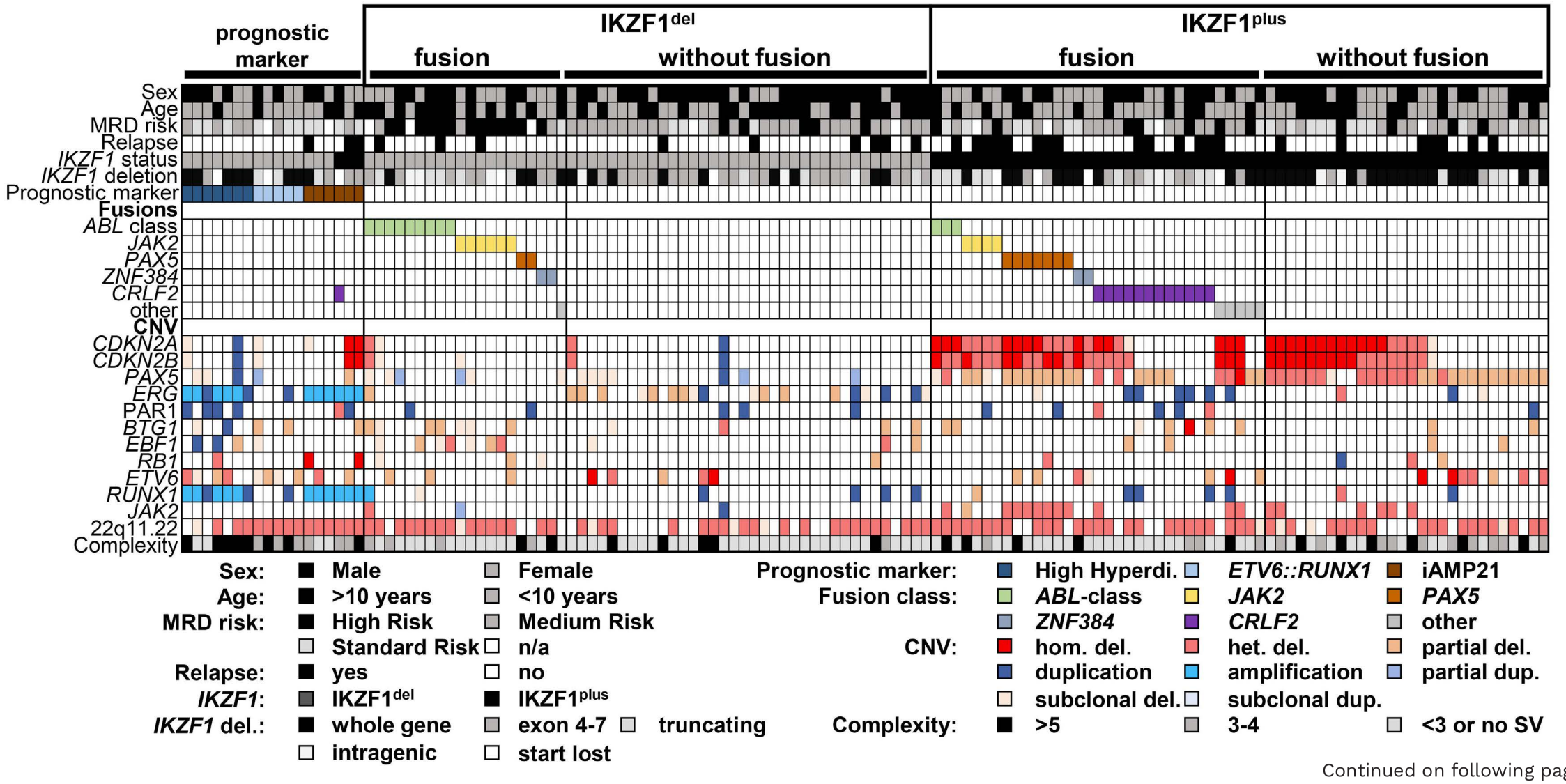

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Figure 1. Overview and Optical Genome Mapping results of the analyzed cohort. (A) A total of 142 patients from the AIEOP-BFM ALL 2000/2009 trials were enrolled. Conclusive results were obtained from 138 patients; 3 patients were excluded. Based on the Optical Genome Mapping (OGM) findings, 5 patients were reclassified. An established prognostic marker (high hyperdiploidy, *ETV6::RUNX1* or iAMP21) was identified in 18 patients and a gene fusion was discovered in 53 patients. (B) Heatmap of OGM results of the 135 analyzed IKZF1^{del} and IKZF1^{plus} patients. Patients were grouped into those with a prognostic marker (high hyperdiploidy, *ETV6::RUNX1*, iAMP21), IKZF1^{del} with or without gene fusion and IKZF1^{plus} with or without gene fusion. MRD: minimal residual disease; CNV: copy number variation; HR: high risk; MR: medium risk; SR: standard risk; n/a: not available; hom. del.: homozygous deletion; het. del.: heterozygous deletion; dup.: duplication; SV: structural variant.

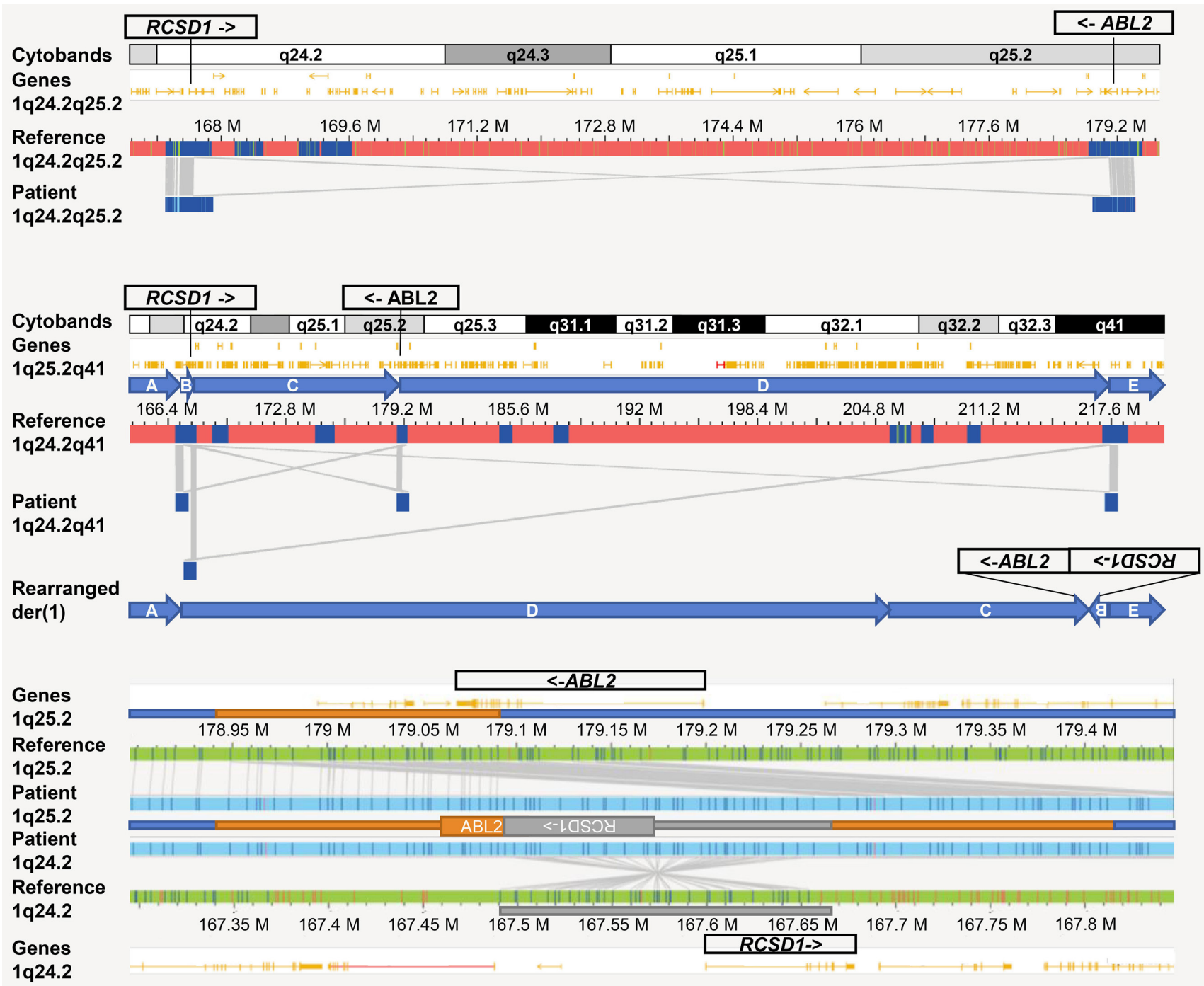

Figure 2. *RCSD1::ABL2* fusion resulting from three distinct structural variants detected by Optical Genome Mapping. (A) Simple inversion inv(1)(q24.2q25.2) with breakpoints in *RCSD1* and *ABL2*. Upper track: cytobands. Second track: genes. Third track: reference genome chromosome 1q24.2-q25.2. Lower track: patient maps indicating inversion with breakpoints in q24.2 and q25.2. (B) Derivative chromosome 1 containing three inversions: der(1)inv(1)(q24.2q25.2)inv(1)(q24.2q41)inv(1)(q25.2q41). Upper track: cytobands. Second track: genes (orange) and segments according to genomic breakpoints (blue). Third track: reference genome chromosome 1q24.2-q41. Fourth track: patient maps indicating inversions. The bottom track shows a schematic representation of the inverted segments. (C) Complex event comprising an ~150 kbp duplication and an ~150 kbp insertion in inverted direction: der(1)dup(1)(q25.2q25.2)ins(1;1)(q25.2;q24.2q24.2). The references for chromosome 1q25.2 (upper bar) and 1q24.2 (lower bar) are shown in green and the corresponding patient maps in light blue. The duplicated region with a breakpoint in *ABL2* is depicted in orange, while the inserted segment with a breakpoint in *RCSD1* is represented in gray.

experience a relapse (Figure 3D). In 45.3% (53/117) of patients without an established marker (HeH, *ETV6::RUNX1*, and iAMP21), a gene fusion was detected by OGM. Patients with a gene fusion had a statistically significant inferior 5-year EFS compared to patients without a fusion (0.56±0.07 *vs.* 0.73±0.06), while the CIR was just slightly increased

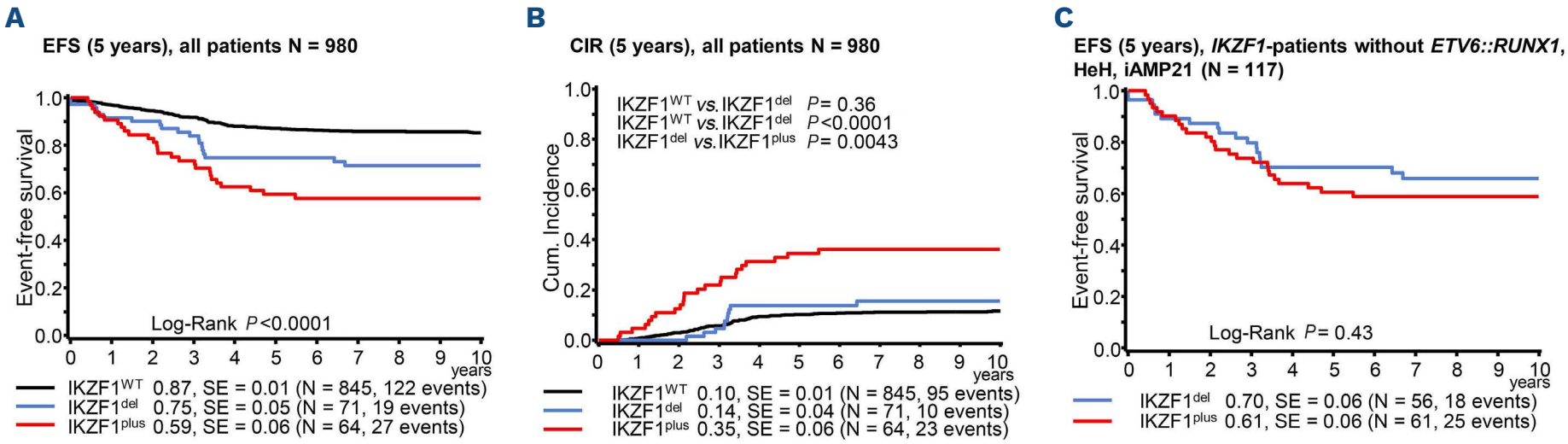

Figure 3. Kaplan-Meier plots for event-free survival and relapse incidence in IKZF1del and IKZF1^{plus} patients. (A) 5-year event-free survival (EFS) and (B) 5-year relapse incidence (CIR) of IKZF1^{del} and IKZF1^{plus} patients compared with 845 ALL-BFM 2000 patients with no *IKZF1* deletion (IKZF1^{WT}). (C) 5-year EFS and (D) 5-year CIR of IKZF1del and IKZF1^{plus} patients when patients with high hyperdiploidy (HeH), *ETV6::RUNX1*, and iAMP21 were excluded from the analysis. (E) 5-year EFS and (F) 5-year CIR of IKZF1^{del} and IKZF1^{plus} patients when patients with HeH, *ETV6::RUNX1*, iAMP21 and gene fusions were excluded from the analysis. (G) 5-year EFS and (H) 5-year CIR of IKZF1$^{del/plus}$ patients (without HeH, *ETV6::RUNX1*, iAMP21) with a gene fusion compared to patients without a gene fusion. (I) 5-year EFS and (J) 5-year CIR for the different fusion subgroups: *ABL*-class, *CRLF2, JAK2, PAX5, ZNF384* and other fusions.

(0.31±0.07 *vs*. 0.20±0.05) (Figure 2G, H). A particularly dismal EFS was observed in patients with *ABL*-class, *PAX5*, and *CRLF2* fusions (Figure 3 I, J). Patients with other or no fusions had the most favorable outcomes. Due to the limited number of samples in each group, no conclusions could be drawn from the stratification of fusion subgroups for IKZF1^{del} and IKZF1^{plus}.

To evaluate the genuine prognostic relevance of the IKZF1^{plus} profile relative to patients solely harboring an *IKZF1* deletion, we analyzed the outcome omitting those patients harboring HeH, *ETV6::RUNX1*, iAMP21, and gene fusions (Figure 3E, F). The disparity in 5-year EFS between IKZF1^{plus} and IKZF1^{del} was then negligible (0.68±0.09 *vs*. 0.77±0.09). In addition, the observed tendency for a higher likelihood of relapse in the IKZF1^{plus} group (0.25±0.09 *vs*. 0.15±0.06) did not reach statistical significance.

Among the 36 IKZF1^{del} and 28 IKZF1^{plus} patients without known prognostic markers (HeH, *ETV6::RUNX1*, iAMP21, gene fusion excluded), 4 IKZF1^{del} and 11 IKZF1^{plus} were categorized MRD standard risk, 25 IKZF1^{del} and 12 IKZF1^{plus} as medium risk, and 5 IKZF1^{del} and 4 IKZF1^{plus} as high risk. For 3 patients, MRD data was unavailable. Given the restricted sample size within each group, a statistical analysis of EFS and CIR was not conducted.

Consistent with recent studies comparing OGM to conventional cytogenetic techniques, a high overall concordance was achieved when comparing the OGM results to the existing MLPA data.[8,9] With the introduction of the IKZF1^{plus} profile, the prognostic impact of the *IKZF1* deletion and accompanying lesion in *BCR::ABL1*-negative BCP-ALL patients was further refined.[7] In this study, we used OGM to perform a genome-wide evaluation of aneuploidies and SV in 97 patients from the AIEOP-BFM ALL 2000 trial, on which the IKZF1^{plus} profile had initially been established, and 45 patients with IKZF1$^{del/plus}$ from the subsequent AIEOP-BFM ALL 2009 trial. Within this cohort, distinct classes of gene fusions were detected in 28.2% of IKZF1^{del} cases and 51.6% of IKZF1^{plus} cases. Consistent with existing literature, patients harboring a gene fusion, specifically *BCR::ABL1*-like fusions (*ABL*-class, *CRLF2*, and *JAK2* fusions), exhibited an inferior 5-year EFS, suggesting their potential function as leukemic drivers.[10] *IKZF1* deletions are frequently observed in *BCR::ABL1*/-like B-ALL, which aligns with the finding that the majority of the detected fusions were classified as *BCR::ABL1*-like.[6,10,11] Interestingly, the 12 patients who carried favorable prognostic markers (HeH, *ETV6::RUNX1*) were exclusively found in the IKZF1^{del} subgroup and did not experience adverse events. It has recently been reported that an *IKZF1* deletion negatively affected the prognosis in patients with HeH but not in those with *ETV6::RUNX1*.[12] When patients with HeH, *ETV6::RUNX1*, and also the unfavorable iAMP21 were excluded from the analysis, the poor prognostic value of the *IKZF1* deletion became more pronounced, and the difference in EFS between IKZF1^{del} and IKZF1^{plus} diminished. Furthermore, when the newly detected gene fusions were excluded from the analysis as well, the prognostic value of IKZF1^{plus} and IKZF1^{del} became comparable but still inferior to IKZF1WT (EFS 0.68±0.09 *vs*. 0.77±0.07 *vs*. 0.87±0.01). *IKZF1* lesions could have an independent prognostic effect, as it has been demonstrated that IKZF1 is implicated in multiple pathways essential for lymphoid cell differentiation and represents a potential therapeutic target.[13] These findings align with previous studies, which have reported modest non-statistically significant differences between IKZF1^{del} and IKZF1^{plus}, but have confirmed the prognostic value of the deletion itself.[14,15] However, it is important to note that this cohort is limited, consisting of a small sample size, and only aneuploidies, CNV and SV were analyzed. Moreover, single nucleotide variants affecting, for example, the Ras and JAK-STAT pathways that are often subclonal were not investigated.[1,3] Nevertheless, the sample size of IKZF1$^{del/plus}$ was comparable to that of the initial study.[7]

Our findings demonstrate that the prognostic relevance of the IKZF1^{plus} profile is not as pronounced as previously anticipated when the patients are genetically better characterized, but that the *IKZF1* deletion itself still exerts a negative prognostic effect.

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Disclosures

No conflicts of interest to disclose.

Contributions

MSt and DS are responsible for study concept. JLL, MZ, WH, AKB, MSt and DS are responsible for study validation. JLL, MZ and DS are

responsible for the formal analysis. JLL, MSt and DS are responsible for the investigation. MZ, WH, AKB, AM, GC, MSc, BS, MSt and DS provided resources. JLL, MZ, MSt and DS are responsible for data curation. JLL wrote the original draft. JLL, MZ, WH, MSc, BS, MSt and DS wrote, reviewed and edited the paper. JLL and MZ are responsible for visualization. MSt, BS and DS supervised the study. JLL and DS are responsible for project administration. MSt and DS are responsible for funding acquisition.

Data-sharing statement

For original data, please contact Steinemann.Doris@mh-hannover.de

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