Challenging Conventional Diagnostic Methods by Comprehensive Molecular Diagnostics: A Nationwide Prospective Comparison in Children With ALL

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ABSTRACT

PURPOSE Treatment stratification in ALL includes diverse (cyto)genetic aberrations, requiring diverse tests to yield conclusive data. We optimized the diagnostic workflow to detect all relevant aberrations with a limited number of tests in a clinically relevant time frame.

METHODS In 467 consecutive patients with ALL (0-20 years), we compared RNA sequencing (RNAseq), fluorescence in situ hybridization (FISH), reverse transcriptase polymerase chain reaction (RT-PCR), karyotyping, single-nucleotide polymorphism (SNP) array, and multiplex ligation-dependent probe amplification (MLPA) for technical success, concordance of results, and turnaround time.

RESULTS To detect stratifying fusions (*ETV6*::*RUNX1*, *BCR*::*ABL1*, ABL-class, *KMT2A*r, *TCF3*::*HLF*, *IGH*::*MYC*), RNAseq and FISH were conclusive for 97% and 96% of patients, respectively, with 99% concordance. RNAseq performed well in samples with a low leukemic cell percentage or low RNA quality. RT-PCR for six specific fusions was conclusive for >99% but false-negative for six patients with alternatively fused exons. RNAseq also detected gene fusions not yet used for stratification in 14% of B-cell precursor-ALL and 33% of T-ALL. For aneuploidies and intrachromosomal amplification of chromosome 21, SNP array gave a conclusive result in 99%, thereby outperforming karyotyping, which was conclusive for 64%. To identify deletions in eight stratifying genes/regions, SNP array was conclusive in 99% and MLPA in 95% of patients, with 98% concordance. The median turnaround times were 10 days for RNAseq, 9 days for FISH, 10 days for SNP array, and <7 days for MLPA and RT-PCR in this real-world prospective study.

CONCLUSION Combining RNAseq and SNP array outperformed current diagnostic tools to detect all stratifying genetic aberrations in ALL. The turnaround time is <15 days matching major treatment decision time points. Moreover, combining RNAseq and SNP array has the advantage of detecting new lesions for studies on prognosis and pathobiology.

ACCOMPANYING CONTENT

Data Supplement

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INTRODUCTION

ALL is the most frequent type of cancer in children and young adults. The primary immunophenotypic distinction is between T-cell (T-ALL) and B-cell precursor (BCP)-ALL lineages. The International Consensus Classification (ICC) distinguishes 22 entities and five provisional entities in BCP-ALL, and three entities and eight provisional entities in T-ALL.[1] The first aberration to be recognized in leukemia was the Philadelphia chromosome encoding the *BCR*::*ABL1* fusion gene, which is a biomarker for tyrosine kinase inhibitor treatment since 2004.[2,3] Over the years, more gene fusions and deletions were added to guide treatment stratification in addition to measurable residual disease (MRD).[4-10] Retrospective analyses using next-generation sequencing identified novel ALL subtypes, including rearrangements of a target oncogene with multiple partner genes, some of which were found to be associated with outcome.[11-14] A new subtype with ABL-class fusions was discovered, driven by fusions of *ABL1* (apart from *BCR*::*ABL1*), *ABL2*, *CSF1R*, or *PDGFRB*, each

CONTEXT

Key Objective

We study whether the agnostic methods whole-transcriptome RNA sequencing (RNAseq) and single-nucleotide polymorphism (SNP) array can replace the classic diagnostic methods karyotyping, fluorescence in situ hybridization (FISH), and multiplex ligation-dependent probe amplification (MLPA) to detect all required risk-stratifying aberrations in pediatric ALL.

Knowledge Generated

RNAseq and FISH were similarly conclusive (96%-97%) with concordant fusion gene detection for 430 (99%) of 435 patients. SNP array was superior in conclusiveness (99%) compared with karyotyping (64%) with concordant aneuploidy detection results for 296 (99%) of 298 patients. SNP array was more sensitive than MLPA for the detection of ALL-relevant gene deletions, and these methods were concordant to determine copy number alteration risk in 296 (98%) of 301 patients.

Relevance

The detection of all genetic aberrations needed for clinical decision taking in the treatment of children with ALL using RNAseq and SNP array is feasible, accurate, and cost effective in a prospective diagnostic setting.

with up to 10 partner genes, with poor outcome and sensitivity to tyrosine kinase inhibition.[15,16]

In the current ALLTogether1 protocol, which enrolls newly diagnosed patients from age 0 to 45 years from 14 European countries (ClinicalTrials.gov identifier: NCT04307576), fusions of eight target genes, three aneuploidy states, and copy number aberrations in eight genes/regions are used for stratification.[10,17] Depending on their role in treatment stratification, these abnormalities need to be identified with turnaround times of 7, 15, or 78 days. The genetic guidelines of ALLTogether1 recommend single-nucleotide polymorphism (SNP) array to detect aneuploidies and SNP array or the targeted multiplex ligation-dependent probe amplification (MLPA) assay to detect focal copy number aberrations. Fluorescence in situ hybridization (FISH) is recommended to detect fusion genes (*ETV6*::*RUNX1*, *BCR*::*ABL1*, *TCF3*::*PBX1* and *TCF3*::*HLF*) and rearrangements involving *KMT2A*, *MYC*, and the ABL-class genes. FISH with a probe for *RUNX1* can be used to screen for intrachromosomal amplification of chromosome 21 (iAMP21), preferably confirmed by DNA array.

Although next-generation sequencing techniques, such as whole-genome sequencing, whole-exome sequencing, and transcriptome sequencing, are increasingly being applied in research, few diagnostic centers validated these methods for clinical routine diagnostics. In our ISO15189-accredited laboratory, RNA sequencing (RNAseq) was validated for fusion gene detection.[18] SNP array and RNAseq are agnostic methods: no previous knowledge is required to select the right diagnostic assay, and they allow genome-wide detection of genetic alterations with high sensitivity.

We performed a prospective comparison of different diagnostic methods to reach a reliable and cost- and labor-effective strategy for the detection of stratifying genetic aberrations in ALL.

METHODS

Patients

Patients (age 0-20 years) were newly diagnosed with ALL at the Princess Máxima Center for pediatric oncology between December 2019 and October 2023. Patients or their parents/guardians gave written informed consent, in accordance with the Declaration of Helsinki, to use their data for research purposes. The Máxima Biobank research protocol was evaluated as a noninterventional study by the Board of the Medical Ethics Committee NedMec (Utrecht, the Netherlands) and the Biobank, and Data Access Committee approved this project. During the inclusion period, patients with *BCR*::*ABL1*-positive ALL were enrolled on the EsPhALL2017/COGAALL1631 trial. Patients with non–*BCR*::*ABL1*-positive ALL were enrolled on Dutch Childhood Oncology Group (DCOG)-ALL11 till July 2020 and on ALLTogether1 from July 2020 onward. Infants were treated according to the Interfant-06 protocol and *KMT2A*-rearranged infants per 2023 in Interfant-21.

Diagnostic Methods

FISH, karyotyping, RNAseq, SNP array, and reverse transcriptase polymerase chain reaction (RT-PCR) were performed for all patients with the available material. MLPA was performed till May 2023. Details on the diagnostic methods are described in the Data Supplement (Methods and Table S1). All diagnostic procedures were performed in the Diagnostic Laboratory of the Princess Máxima Center or in the Genome Diagnostics Laboratory of University Medical Center Utrecht under ISO15189 accreditation. The percentage of

leukemic cells was determined by flow cytometry after Ficoll separation.

Subtype Assignment

Determination of final ALL subtype according to ICC[1] was performed in a clinical setting by molecular and genetic staff who performed weekly review of cases. Stratifying gene fusions and ploidy aberrations as well as the copy number alteration risk group on the basis of deletions of eight genes/regions were reported. A risk-stratifying fusion overruled high hyperdiploidy.

RESULTS

Patient Characteristics

In the Netherlands, approximately 110 children and adolescents are diagnosed with ALL each year. Until 2018, cytogenetic diagnostics was performed at the local academic hospital where the patient was treated, whereas the DCOG laboratory in The Hague performed central molecular diagnostics. From 2018, all patients and molecular diagnostics are centralized at the Princess Máxima Center for pediatric oncology, resulting in a single workflow for patient material (Data Supplement, Fig S1). Cytogenetics and SNP array are performed at the neighboring University Medical Center Utrecht. The 467 consecutive patients included in the comparison cohort were diagnosed between December 2018 and October 2023, with diagnostic methods performed in parallel (Fig 1; Data Supplement, Fig S2). During this period, the number of stratifying aberrations increased and the turnaround time for diagnostic results decreased (Data Supplement, Table S2). The median age of the cohort was 5.6 years (range, 0-20), 55.7% were male, and the immunophenotypes were 87.2% and 12.8% for B- and T-lineage, respectively. In this prospective ALL cohort, we compared the diagnostic results, in particular the percentage of patients with a conclusive assay, the sensitivity to detect all stratifying aberrations, and the turnaround time.

Conclusiveness and Turnaround Time

The median leukemic cell percentage of the diagnostic samples was 91% (range, 4%-99%), and 92% of the samples had ≥60% leukemic cells, which is sufficient for the detection of clonal deletions by MLPA. Only 14 samples had a leukemic cell percentage below 30%, possibly resulting in lower sensitivity to detect alterations by FISH or RNAseq. The different diagnostic methods to detect gene fusions, ploidy, and copy number alterations yielded a conclusive result for ≥95% of the samples, except for karyotyping (64%), which was often cryptic or showed normal karyotype, which was regarded as noninformative, likely due to nonmitosis of leukemic cells (Table 1). The turnaround times for FISH, RT-PCR, and MLPA were within 15 days for all samples. For SNP array, the median turnaround time was 10 days, with 99.7% within 15 days (one sample had to be repeated), and for RNAseq with two runs per week, the median turnaround time was 10 days, with 95% within 15 days (observation period July-October 2023, 56 patients). Importantly, assay failures for RNAseq (12 patients) and FISH (20 patients) did not overlap, suggesting FISH as a backup in case RNAseq is not successful.

Fusion Gene Detection

RNAseq and interphase FISH were used to detect fusions or rearrangements of eight target genes. These methods gave conclusive results for 97% and 96% of the cohort, respectively, resulting in 435 patients with overlapping data (Table 1). The concordance for the detection of stratifying fusions (*ETV6*::*RUNX1*, *BCR*::*ABL1*, ABL-class, *KMT2A*r, *TCF3*::*HLF*/*PBX1*, and *MYC*r) was 99% (Data Supplement, Table S3A). Chimeric transcripts were detected by RNAseq in samples with leukemic cell percentages as low as 10% and RNA integrity scores as low as 2. The few discordant results were explained by inherent limitations of the methods used. The *TCF3*::*HLF* fusion in one patient was not detected by routine analysis of RNAseq data due to the insertion of intronic sequences, which was previously described to occur for this gene fusion.[19] The insertion resulted in a cryptic exon that hampered the mapping algorithm of the StarFusion pipeline. To avoid missing *TCF3*::*HLF* fusions, we included *HLF* expression level in the RNAseq report followed by inspection of *HLF* exon coverage in suspected cases (Data Supplement, Fig S3). The *KMT2A*::*USP2* fusion was not detected by FISH because of an inversion on 11q23 between the FISH probes that does not result in an aberrant hybridization pattern. Notably, two *TNIP1*::*PDGFRB* ABL-class fusions detected by RNAseq were initially missed by FISH because of subclonality (10%-15%) but detected upon re-evaluation of the FISH data. One sample with a *BCR*::*ABL1* fusion was missed by FISH, possibly because of an underlying insertion rather than a translocation, but was detected by imbalance at the breakpoints on SNP array. RT-PCR was performed for a limited number of fusion genes that were historically used for the detection of canonical subtypes in ALL. RT-PCR gave concordant results except for samples with alternative exons involved in the fusion (Data Supplement, Table S3A). Chromosome banding analysis showed that karyotypes of *ETV6*::*RUNX1* and ABL-class fusions were mostly cryptic or noninformative, and karyotypes were mostly confirmative for *BCR*::*ABL1*, *TCF3*::*PBX1*, and *KMT2A* rearrangements (Data Supplement, Table S3A). In summary, both RNAseq and FISH were highly informative to detect stratifying fusion genes. Notably, RNAseq detected additional fusion genes and performed well in samples with a low leukemic cell percentage or low RNA quality.

Ploidy and iAMP21 Detection

SNP array and karyotyping were compared for the detection of iAMP21, high hyperdiploidy, low hypodiploidy, and near-haploidy in 298 patients (Table 1; Data Supplement, Table S3B). SNP array is considered the standard method for the

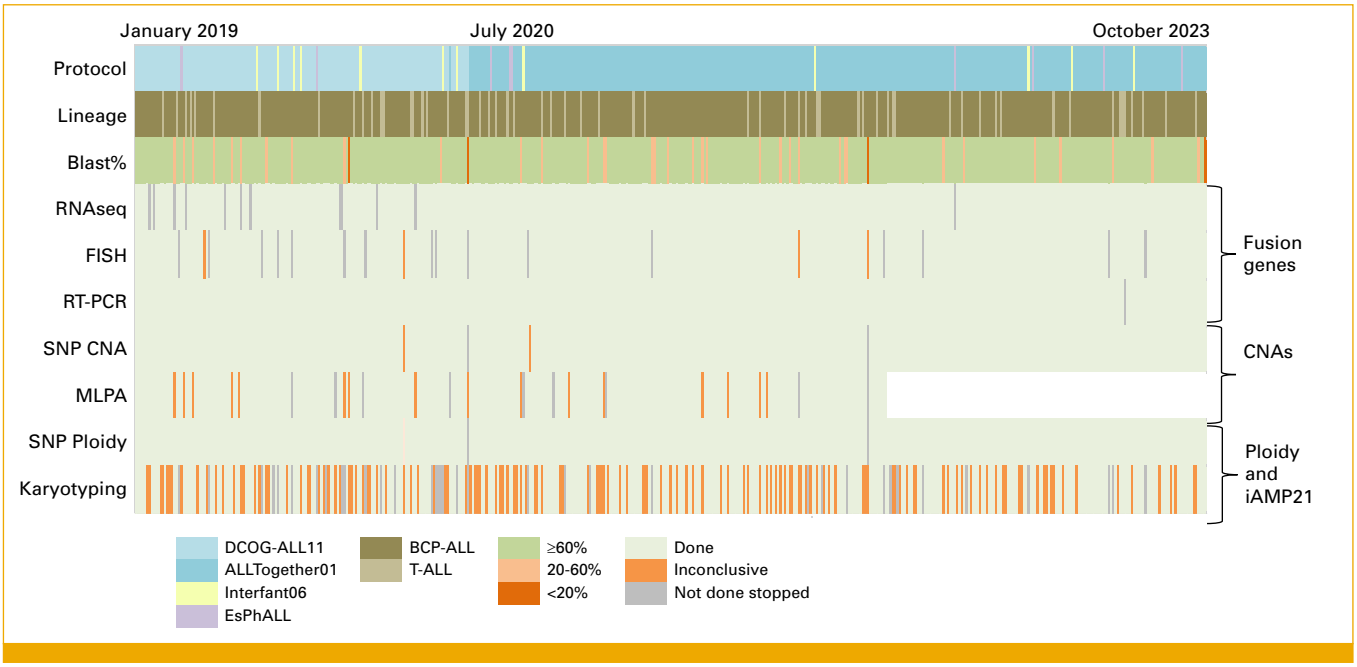

FIG 1. Cohort used for comparison: 467 newly diagnosed pediatric ALL. Metadata per patient sample is visualized in columns. Protocol includes DCOG-ALL11 (light blue), ALLTogether01 (mid-blue), EsPhALL (purple), and treated as Interfant06 (yellow). Lineage was BCP-ALL (dark green) or T-ALL (light green). Blast percentage was categorized as above 60% (green), between 20% and 60% (orange), and below 20% (red). The diagnostic assays applied were categorized as successful (light green), inconclusive (orange), not done (gray), or discontinued (white). BCP, B-cell precursor; CNA, copy number alteration; DCOG, Dutch Childhood Oncology Group; FISH, fluorescence in situ hybridization; iAMP21, intrachromosomal amplification of chromosome 21; MLPA, multiplex ligation-dependent probe amplification; RT-PCR, reverse transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.

detection of ploidy changes because it detects loss of heterozygosity (LOH). In our cohort, one-fourth low-hypodiploid and one-third near-haploid cases showed high-hyperdiploid karyotypes, suggesting that SNP array was essential for the detection of high-risk aneuploidy in two (28.6%) of seven patients (Data Supplement, Fig S4). On the basis of the characteristic amplification pattern on SNP array,[20-22] 13 iAMP21 cases were identified. Using a combination of karyotyping and *RUNX1* metaphase FISH, nine of 13 iAMP21 cases were confirmed, with the remaining four cases lacking informative karyotypes. In summary, SNP array outperforms conventional karyotyping and FISH, and is sufficient to detect all required risk-stratifying aneuploidies and iAMP21. In addition, SNP array detected triploidy, near-tetraploidy, copy-neutral LOH, and gains and losses in the ALL genomes (Fig 2).

Copy Number Alteration Detection

SNP array and MLPA were compared for the detection of copy number alterations in *IKZF1*, *CDKN2A/B*, *PAX5*, *EBF1*, *ETV6*, *RB1*, *BTG1*, and the PAR1 region in 301 samples (Table 1). The results were evaluated according to the ALLTogether1 genetic guidelines, requiring at least two adjacent MLPA probe locations to support a deletion and a clonality of ≥40%. The concordance for alterations was 96%, with 98% of the samples assigned to the same copy number alteration risk group (Data Supplement, Table S3C). MLPA was not done or inconclusive for half of the samples with <60% leukemic cells (15 samples). MLPA showed false-positive results in a case with a noisy profile and a case with extreme hyperdiploidy, and false-negative results due to ploidy-related normalization issues or subclonal deletions (Data Supplement, Fig S5). SNP array may miss small deletions, especially when the probe coverage is low. For five cases with an *ETV6* exon 1 deletion detected by MLPA (two probes), the lack of SNP array probes on the Infinium CytoSNP-850K array resulted in a false-negative result in one case with a very focal deletion and inconclusive results in three cases, although deletion of the region upstream was suggestive of exon 1 deletion (Data Supplement, Fig S6). Our comparison showed that SNP array is more reliable than MLPA in aneuploid samples and samples with low leukemic cell percentage and can replace MLPA to detect copy number alterations if we take the *ETV6* exon 1 issue into account. Moreover, SNP array better distinguishes mosaicism in subclones from low leukemic cell percentage if at least one clonal aberration is present. Beyond the currently required detection of MLPA-based deletions, SNP array detected deletions in (single) exons and aberrations in low mosaicism (20%), providing options for future evaluation of their prognostic value. Finally, SNP array detected recurrent deletions in additional genes, including *SH2B3*, *NF1*, *TBL1XR1*, and *TP53* (Fig 3).

TABLE 1. Conclusiveness, Turnaround Time, and Concordance of Diagnostic Results

Technique	Conclusive Test	Turnaround Time Median (range)[a]	Aberrations Detected (number)	Overlap for Comparison and Concordance
RNA sequencing	455/467 (97%)	16 days (7-38) 1 run/wk 10 days (7-15) 2 runs/wk	138 stratifying fusion genes[b] 57 subtype-defining fusions[c] 39 other fusions (including *CRLF2*)	435 patients compared for fusion detection 430 concordant (99%)
FISH	447/467 (96%)	9 days (4-15)	128 stratifying fusion genes[b] 15 subtype-defining fusions[c] 14 other break-apart	
RT-PCR	466/467 (>99%)	<7 days	118 stratifying fusions 15 subtype-defining fusion genes	
SNP array ploidy	464/467 (99%)	10 days (5-18)	139 HeH, 7 NH/HoL, 13 iAMP21	298 patients compared for ploidy detection 296 concordant (99%)
Chromosome banding (+FISH *RUNX1*)	300/467 (64%)	18 days (15-20)[d]	119 HeH, 3 NH/HoL, 9 iAMP21	
SNP array CNA	463/467 (99%)	10 days (5-18)	463 CNA risk	301 patients compared for copy number alterations 290 concordant genes (96%) 296 concordant CNA risk (98%)
MLPA CNA	303/319 (95%)	<7 days	303 CNA risk	

Abbreviations: CNA, copy number alteration; FISH, fluorescence in situ hybridization; HeH, high hyperdiploid (51-67 chromosomes); HoL, low hypodiploid (30-39 chromosomes); iAMP21, intrachromosomal amplification of chromosome 21; MLPA, multiplex ligation-dependent probe amplification; NH, near-haploid (<30 chromosomes); RT-PCR, reverse transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.

[a]For routine practice, samples are batched for weekly or biweekly runs and analyses to optimally balance efficient assay volume and turnaround time. Turnaround times for a single assay can be shorter when a speed diagnosis is required.

[b]Stratifying fusions: *ETV6*::*RUNX1*, *BCR*::*ABL1*, ABL-class gene fusion, *TCF3*::*HLF*, *KMT2A* rearrangement, *MYC* rearrangement.

[c]Subtype-defining fusions not used for stratification: *TCF3*::*PBX1*, rearrangements of *DUX4*, *PAX5*, CEBP family genes, JAK-class genes, *MEF2D*, *ZNF384*.

[d]Karyotyping was not prioritized for a short turnaround time during the observation period.

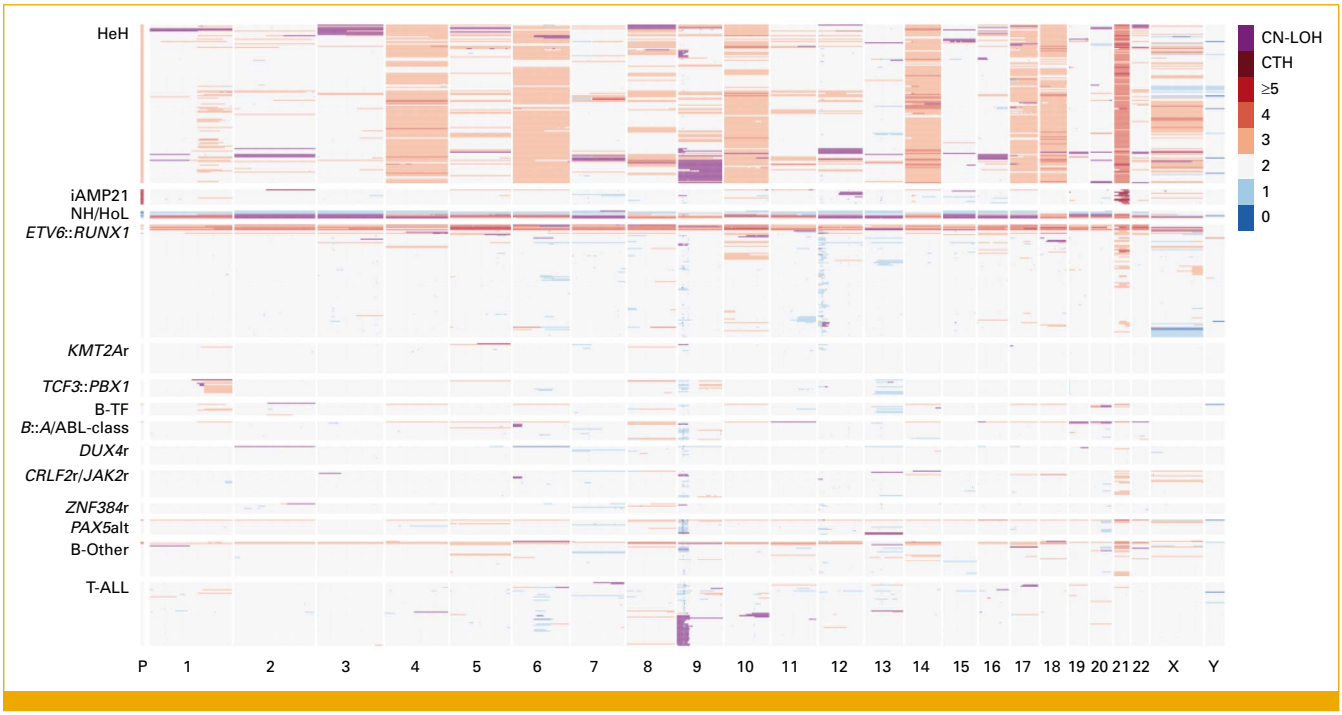

FIG 2. Chromosomal abnormalities in pediatric ALL cohort. Copy number data derived from SNP array per patient ordered by main subtype (rows) and 1-Mb bins per chromosome (columns). Color scale from blue to red represents zero to ≥five copies. Brown, CTH; purple, CN-LOH. The first column, labeled P, indicates the ploidy of the sample. *B*::*A*, *BCR*::*ABL1*; B-TF, BCP-ALL driven by transcription factor aberration; CN-LOH, copy-neutral loss of heterozygosity; CTH, chromothripsis; HeH, high hyperdiploid; HoL, low hypodiploid; iAMP21, intrachromosomal amplification of chromosome 21; NH, near-haploid; SNP, single-nucleotide polymorphism.

Added Value of RNAseq

RNAseq identified subtype-defining alterations beyond the obligatory stratifying fusion genes. First, RNAseq detected novel fusion-defined subtypes of a target gene with multiple partners, such as rearrangements of *ZNF384* and *MEF2D*. Second, RNAseq yielded exact information on the genes and exons involved in the fusion, allowing, for example, to determine the kinase and fusion partner in ABL-class fusions, where FISH does not distinguish between breakpoints in the neighboring *PDGFRB* and *CSF1R* genes. This information can also be used to develop fusion read-driven MRD PCR.[23-26] Third, using gene expression levels identified patients with BCP-ALL with likely rearrangements of the *IGH* locus, leading to overexpression of *DUX4*, *CRLF2*, *MYC*, *CEBPA*, and *CEBPB* (Data Supplement, Fig S7) as well as patients with T-ALL with likely rearrangements of *TLX1* or *TLX3*. Fourth, subtypes defined by a specific pathogenic single-nucleotide variant such as *PAX5* p.Pro80Arg and *IKZF1* p.Asn159Tyr could be detected in RNA sequence reads, as well as other mutations in expressed genes (eg, RAS pathway genes and *NOTCH1*; Fig 3).

Genetic Subtypes in Pediatric ALL

Among 407 patients with BCP-ALL, we identified 19 subtypes at frequencies similar to those previously described.[27] Seven patients with BCP-ALL had a rare fusion gene, such as *TCF3*::*FLI1*. Excluding two patients with low leukemic cell percentage, 22 patients with BCP-ALL (5%) remained B-other, defined by the absence of a gene fusion or stratifying aneuploidy aberration (Data Supplement, Table S4). In T-ALL, ABL-class (5%) and *KMT2A*-rearranged (3%) were relevant for patient stratification in ALLTogether1. Both *KMT2A* partner genes, *SEPTIN9* and *CBL*, were novel in T-ALL. *KMT2A*::*CBL* was previously described as a rare fusion in acute myeloid leukemia and ALL.[28,29] Furthermore, T-ALL subtypes characterized by rearrangements of *TAL1* (25%), *TLX3* (15%), *TLX1* (7%), and *LMO2* (2%) were identified. In 10% of T-ALL, a rare fusion was identified and in 33% no subtype could be assigned (Data Supplement, Table S5). One rare fusion with therapeutic relevance was *ETV6*::*NTRK3*, which was shown to drive ALL in a knock-in mouse model and showed sensitivity to TRK inhibitors in preclinical models and case reports of both T-ALL and BCP-ALL.[30-33]

Efficient Workflow for ALL Diagnostics

We compared different logistic aspects contributing to the efficiency of diagnostic methods (Table 2). Taking labor, turnaround time, and assay volume into account, we established an optimal workflow: RNAseq and SNP array for each diagnostic ALL sample to identify fusions and ploidy aberrations within 15 days; FISH and RT-PCR restricted to infants (<1 year) to detect *KMT2A* rearrangements within 7 days; and *ETV6*::*RUNX1* FISH for all BCP-ALL to provide

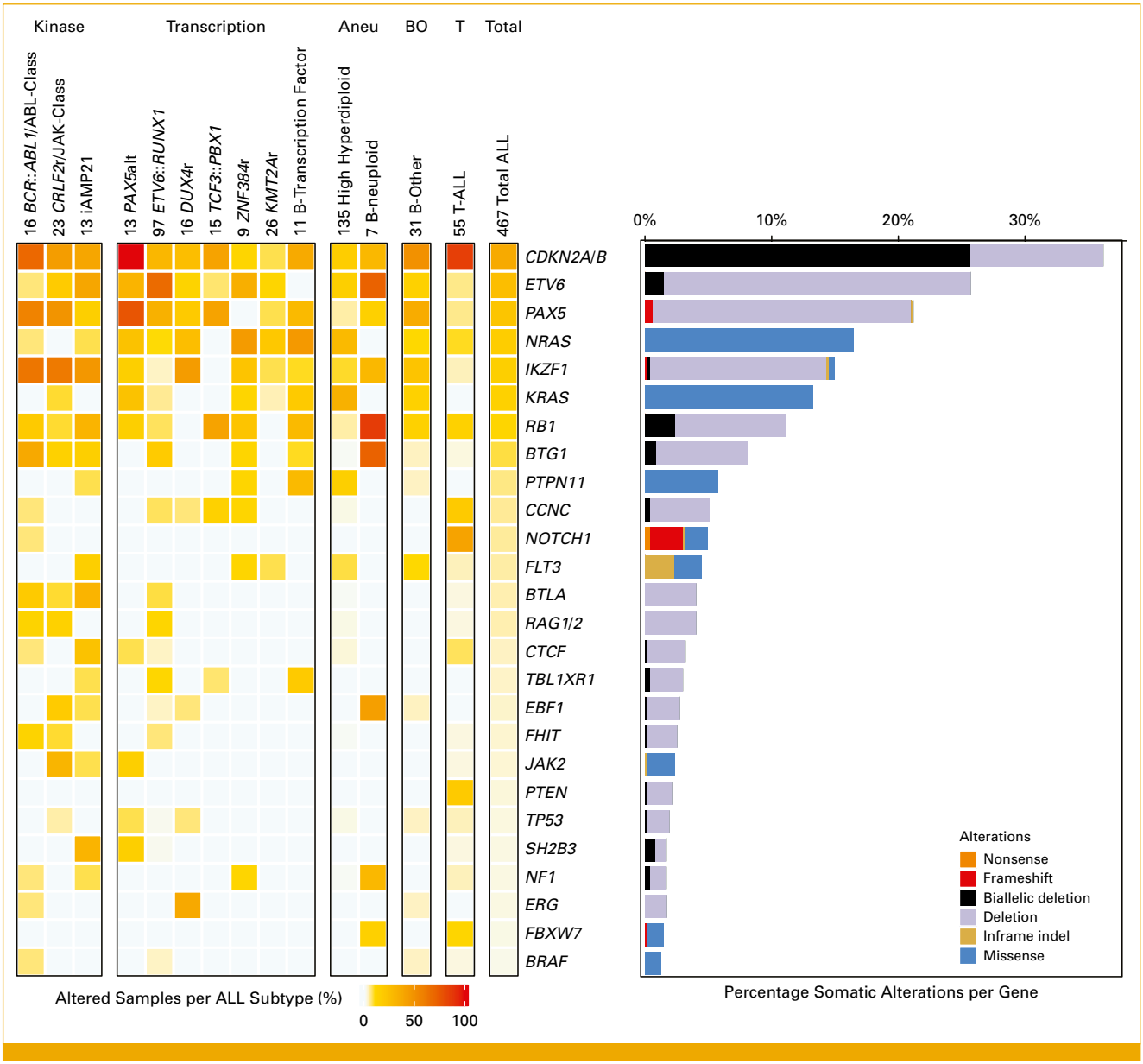

FIG 3. Frequencies of genetic aberrations across ALL subtypes. The frequency and type of pathogenic sequence variants, derived from RNA sequencing, and copy number alterations, derived from SNP array, are shown per ALL subtype. Subtypes were defined as follows: Kinase-driven including *BCR*::*ABL1* and ABL-class fusions, *CRLF2*-rearranged and *JAK*-class fusions, iAMP21; transcription factor–driven including PAX5alt, *ETV6*::*RUNX1*, *DUX4*-rearranged, *TCF3*::*PBX1*, *ZFN384*-rearranged, *KMT2A*-rearranged, remaining BCP-ALL driven by a fusion or a pathogenic variant of a transcription factor (*CEBP*, *MYC*, *PAX5*, *TCF3*::*HLF*, *MEF2D*); aneuploidy-driven including high hyperdiploid, remaining aneuploid BCP-ALL (low hypodiploid, near-haploid); B-Other, including BCP-ALL without subtype-defining fusions or ploidy aberrations; T-ALL, including T-ALL except those with ABL-class fusion or KMT2A rearrangement, which were analyzed together with the corresponding BCP-ALL subtype. The number of cases per (combined) subtype is indicated above the heatmap. Genes are ordered according to the total number of genetic aberrations detected across the total cohort and included if mutated or deleted in >1% of the cases. For genes included in the screen, see the Data Supplement (Table S1). Left panel: Heatmap with genes on the rows and subtypes on the columns showing the percentage of affected samples per subtype. Right panel: Percentage of samples with alterations in each gene, with the alteration type indicated by color. If more than one alteration was present, the alteration highest in the legend list is included. BCP, B-cell precursor; iAMP21, intrachromosomal amplification of chromosome 21; SNP, single-nucleotide polymorphism.

additional evidence for iAMP21. Fixed cells are stored to perform FISH or karyotyping as a backup in case of failed RNAseq or unclear clonality of aberrations (Fig 4). We estimate a cost reduction on the basis of the Dutch health care reimbursement system around 60% (Data Supplement, Table S6).

TABLE 2. Comparison of General Logistic Aspects of Diagnostic Methods

Technique	Detect All Relevant Fusion Genes	Detect All Relevant Ploidy and CNA	Availability of Diagnostic Method[a]	Turnaround Time[b]	Resources for Labor[c]	Costs of Reagents[d]
RT-PCR One assay	No		R	2-5 days	€	€
RNA panel sequencing	Yes		S	10-15 days	€€	€€€
RNA whole-transcriptome sequencing	Yes		S	10-15 days	€€€	€€€
FISH One assay	Yes		R	4-10 days	€€€	€
Chromosome banding analysis	No	No	R	5-20 days	€€€	€
SNP array		Yes	R	10-15 days	€€€	€€
DNA panel analysis (eg, MLPA)		No	R	2-5 days	€€	€
Whole-genome sequencing	Yes	Yes	S	10-20 days	€€	€€€€

NOTE. The table gives an indication of relative investment per method. Exact turnaround time and costs are dependent on an efficient assay volume to perform (bi)weekly batch analysis, laboratory setting, central or decentral facilities, priority choices, reimbursement policy, and so on.

Abbreviations: CNA, copy number alteration; FISH, fluorescence in situ hybridization; MLPA, multiplex ligation-dependent probe amplification; RT-PCR, reverse transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.

[a]Availability: R, routine method, widely available; S, specialized laboratories.

[b]Estimated theoretical turnaround time, not taking sample batches for (bi)weekly runs into account.

[c]Estimated labor resources per batch of samples including analysis: €, 1 day; €€, 2 days; €€€, 3 days.

[d]Estimated costs of reagents per sample per assay: €, €30-100; €€, €100-300; €€€, €300-600; €€€€, €600-3,000.

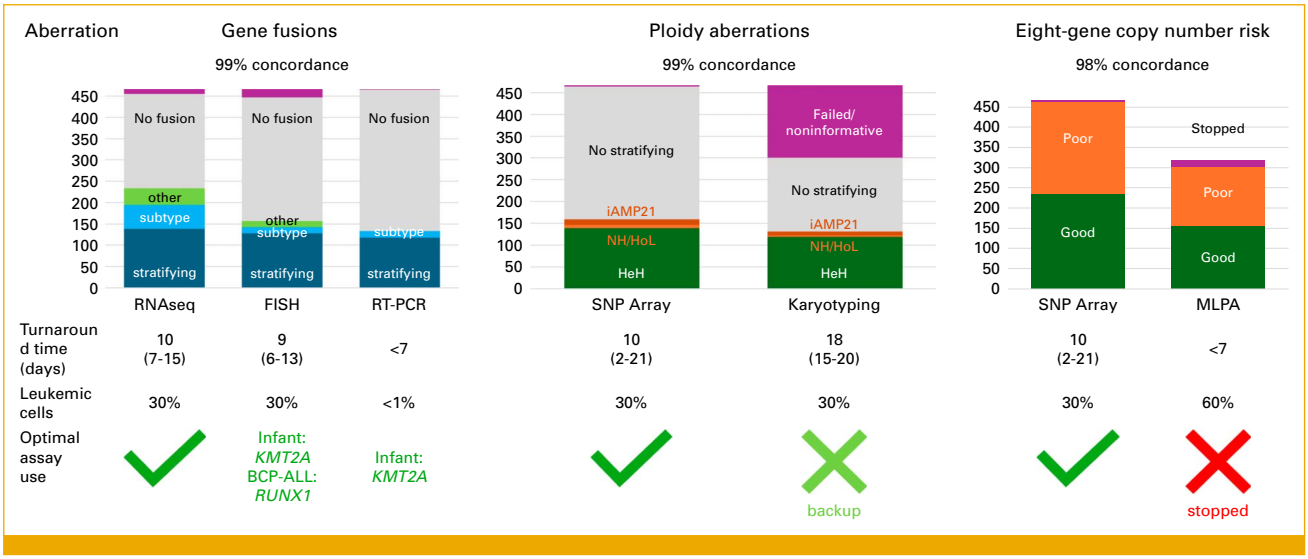

FIG 4. Summary of diagnostic results and optimal assay use. Upper panel: Diagnostic yield and concordance for the detection of gene fusions, ploidy aberrations, and eight-gene/region copy number risk using different diagnostic methods. Colors of gene fusions: dark blue, stratifying fusion; light blue, other subtype-defining fusion; green, other fusion; gray, no fusion; pink, assay failed or not done. Colors of ploidy aberrations: dark green, HeH; orange, NH/HoL, red, iAMP21; gray, no stratifying ploidy aberration; pink, assay failed or noninformative or not done. Colors of eight-gene/region copy number risk: dark green, good risk; orange, poor risk; pink, assay failed or not done. Lower panel: turnaround time of the diagnostic methods in days, median (range); recommended leukemic cell percentage; optimal assay use implemented in our current diagnostic workflow with routine use of RNAseq and SNP array combined with selective use of FISH and RT-PCR. FISH, fluorescence in situ hybridization; HeH, high hyperdiploid; iAMP21, intrachromosomal amplification of chromosome 21; MLPA, multiplex ligation-dependent probe amplification; NH/HoL, near-haploid or low hypodiploid; nucFISH, nuclear FISH; RNAseq, RNA sequencing; RT-PCR, reverse transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.

DISCUSSION

The combination of RNAseq and SNP array provides for a comprehensive diagnostic strategy with a success rate of 97%-99% and a turnaround time within 2 weeks. Given the utmost priority to know whether an infant has a *KMT2A* rearrangement for inclusion in the Interfant-21 protocol, we balance comprehensiveness with speed to detect *KMT2A* rearrangement in infants within 1 week by FISH and RT-PCR. Importantly, we show that RNAseq is sufficient to detect all stratifying fusion genes in a prospective diagnostic lab setting, with real-world challenges including nonoptimal sample conditions such as low leukemic cell percentage or low-quality RNA. Fusion detection by RNAseq performed well in these conditions because we used a whitelist to retain clinically relevant fusion genes when only a few fusion reads were present.[18]

RNAseq yielded additional information on fusions, expressed mutations, and expression levels. Clustering on the basis of gene expression can support subtype detection and identify -like subtypes with a similar expression but lacking the canonical fusions, such as *BCR::ABL1*-like and *ETV6::RUNX1*-like.[27,34,35] Thus, RNAseq results in robust identification of ALL subtypes including those without a chimeric transcript, which is useful for future study of the prognostic value of rare subtypes. Interestingly, systematic screening of all diagnostic pediatric ALL samples for ABL-class fusions revealed that these are more common in T-ALL (5%) than in BCP-ALL (1%). Lack of previous detection of ABL-class fusions in T-ALL is likely due to a screening bias: ABL-class fusions were first described in high-risk BCP-ALL in children and adults,[15,16,36] and *NUP214::ABL1* fusions were detected in episomes in T-ALL and not considered the drivers for the T-ALL. In the ALLtogether1 protocol, both patients with BCP-ALL and T-ALL with ABL-class lesions are eligible for imatinib starting day 15 after initial diagnosis.

SNP array outperformed karyotyping for aneuploidy and iAMP21 detection and MLPA for copy number alteration detection. The sensitivity of SNP array was as low as 20% mosaicism on the basis of B-allele frequency, allowing detection of aberrations in case of subclones or low leukemic cell percentage.

Our comparison showed that the agnostic assays RNAseq and SNP array are justified and adequate if many and diverse stratifying genetic abnormalities need to be determined in a limited turnaround time, as is the case for patients with pediatric ALL. Targeted gene panels suffer from necessary adaptation if novel genes or regions need to be added, and FISH assays require hands-on time which increases with the number of FISH probes to be tested. On the basis of our analyses, karyotyping was never essential for adequate stratification, and therefore, this labor-intensive method can be omitted as standard diagnostic test. As a future sole diagnostic method to detect genomic aberrations in ALL,

whole-genome sequencing has shown high feasibility and utility in retrospective studies.[37,38] The main challenges for its routine diagnostic use are the costs, the turnaround time, and the requirement for germline DNA.

Reducing the number of tests done in a diagnostic laboratory lowers the number of external quality assessment schemes that are needed for ISO15189 accreditation. For example, every FISH or PCR test requires a separate validation, whereas fusion detection by RNAseq is a single validation. Since there is no suitable external quality assessment scheme for fusion gene detection by RNAseq, we have performed yearly academic round-robins with other European laboratories. Replacing most of the targeted diagnostics tests by RNAseq and SNP array has improved the efficiency and reduced the costs of our diagnostic workflow with 50%. However, local and national policies and patient numbers may affect the benefit of this diagnostic strategy.

We conclude that the combination of RNAseq and SNP array accurately detects all stratifying genetic alterations in pediatric ALL with a high technical success rate in a clinically relevant time frame and informs retrospective studies into their role in prognosis and pathobiology.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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