Research Article

Mutated WT1, FLT3-ITD, and NUP98-NSD1 Fusion in Various Combinations Define a Poor Prognostic Group in Pediatric Acute Myeloid Leukemia

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Acute myeloid leukemia is a life-threatening malignancy in children and adolescents treated predominantly by risk-adapted intensive chemotherapy that is partly supported by allogeneic stem cell transplantation. Mutations in the WT1 gene and NUP98-NSD1 fusion are predictors of poor survival outcome/prognosis that frequently occur in combination with internal tandem duplications of the juxta-membrane domain of FLT3 (FLT3-ITD). To re-evaluate the effect of these factors in contemporary protocols, 353 patients (<18 years) treated in Germany with AML-BFM treatment protocols between 2004 and 2017 were included. Presence of mutated WT1 and FLT3-ITD in blasts (n=19) resulted in low 3-year event-free survival of 29% and overall survival of 33% compared to rates of 45-63% and 67-87% in patients with only one (only FLT3-ITD; n=33, only WT1 mutation; n=29) or none of these mutations (n=272). Including NUP98-NSD1 and high allelic ratio (AR) of FLT3-ITD (AR ≥ 0.4) in the analysis revealed very poor outcomes for patients with co-occurrence of all three factors or any of double combinations. All these patients (n=15) experienced events and the probability of overall survival was low (27%). We conclude that co-occurrence of WT1 mutation, NUP98-NSD1, and FLT3-ITD with an AR ≥ 0.4 as triple or double mutations still predicts dismal response to contemporary first- and second-line treatment for pediatric acute myeloid leukemia.

1. Introduction

Pediatric acute myeloid leukemia (AML) is a rare and heterogeneous disorder, for which continuous improvement of risk-adapted treatment approaches over the last 30 years has led to overall survival rates of approximately 70% [1, 2]. In current pediatric AML treatment protocols, cytogenetic abnormalities of the leukemic blasts at initial diagnosis are important indicators for risk group stratification and treatment assignment [1, 2]. Approximately, 25% of pediatric patients have AML blasts with a normal karyotype, but even these cases often harbor somatic mutations in genes such as WILMS TUMOR 1 (WT1), NPM1, NRAS, KRAS, Fms-like tyrosine kinase 3 (FLT3), and/or c-KIT/CD117 [1, 2].

The WT1 gene is located on chromosome 11, has ten exons and four zinc finger domains, and functions as a transcription factor and master regulator of tissue development [3]. Within normal hematopoiesis, WT1 has two distinct roles: in early
toward the myeloid lineage [4]. In AML, WT1 mutations are present in approximately 10% of patients and predominantly located in exons 7 and 9, which contain the DNA-binding zinc finger domains of the protein. The majority of these mutations are out-of-frame deletions/insertions or premature termination codons that will lead to truncated proteins with altered functional consequences for the cells [5]. If these truncated proteins are stable, they might have dominant negative effects by partially blocking the wild-type WT1 protein; if unstable, the diminished WT1 protein levels may lead to haploinsufficiency [5]. Nevertheless, it has been clearly established that the occurrence of WT1 mutations in AML blasts with normal karyotypes is associated with adverse clinical outcomes in adult [6–9] as well as pediatric patients [10, 11].

Somatic WT1 mutations in AML blasts often co-occur with other genetic aberrations, most frequently with an intratumor tandem duplication in the juxta-membrane domain of the tyrosine kinase receptor FLT3 (FLT3-ITD) [5]. Classified as type-1 or proliferating mutation, FLT3-ITDs are present in 10-15% of pediatric AML cases and lead to poor clinical outcomes [12–14]. We previously demonstrated in a cohort of 298 pediatric patients with de novo AML treated before 2004 on AML-BFM protocols that the combination of FLT3-ITD and mutated WT1 is associated with even worse survival [10]. Comparably, an independent study from the Children’s Oncology Group (COG) in a cohort of 842 children with de novo AML showed that the poor prognostic impact of WT1 mutations depends on the FLT3-ITD status [11]. These two pediatric studies confirmed earlier findings in adults that first established the adverse prognostic impact of both WT1 and FLT3-ITD mutations [15, 16].

Two additional prognostic indicators in FLT3-ITD-positive AML cases established in the last few years are the mutational burden in each patient defined as the ratio between mutant and wild-type FLT3-ITD alleles (allelic ratio, AR) [12, 17, 18] and the co-occurrence of FLT3-ITD with a cytogenetically cryptic translocation of chromosomes 5 and 11 or t(5;11)(q35;p15) [19]. This translocation leads to fusion of the nucleoporin (NUP98) gene on chromosome 11 and the gene for nuclear receptor binding SET-domain protein 1 (NSD1) of chromosome 5 (NUP98-NSD1). As the breakpoints for the NUP98 gene are often not detected by classical cytogenetic due to its terminal localization at 11p15, it has been described in AML cases with a “normal” karyotype [20]. Importantly, this rare recurrent aberration is mutually exclusive with other recurrent translocations and more prevalent in pediatric AML, in which it is associated with the presence of FLT3-ITD and poor survival outcomes [21, 22].

In the present study, we re-evaluated the role of mutations in WT1, FLT3-ITD, and the NUP98-NSD1 translocation as prognostic factors in two contemporary pediatric treatment protocols by analyzing their association with co-occurring genetic and cytogenetic aberrations and by determining their clinical significance and influence on treatment outcome. Thereby, we were able to define a group of high-risk patients for which the efforts for salvage/second line treatment largely failed.

2. Materials and Methods

From April 2004 to May 2017, 841 patients aged 0–18 years with de novo AML (excluding Fab M3 and Down Syndrome) were treated in Germany according to the AML-BFM 04 trial (ClinicalTrials.gov Identifier: NCT00113435) or the AML-BFM 2012 registry and trial (EudraCT number: 2013-00001839) (Figure I(a)). Both trials were approved by the ethical committees and institutional review boards of university hospitals of Münster and Hannover and an informed consent was obtained from each patient or their legal guardians before the beginning of treatment. Standard procedures for the diagnosis of AML were carried out by the German AML-BFM reference laboratory as previously described [23–25]. This included mutation analysis in WT1, FLT3-ITD, NPM1, NRAS, and c-KIT by Sanger and/or next-generation sequencing or GeneScan analysis. In 353 patients (42%), sufficient material and clinical data were available for further analysis. As a confirmation, material from WT1 and/or FLT3-ITD positive and negative cases was re-analyzed by next-generation sequencing (NGS) using the TruSight Myeloid Panel (Illumina) with median read counts for WT1 and FLT3-ITD of around 4,200 and 6,000 reads, respectively, as we described previously [27]. In addition, the allelic ratio of FLT3-ITD to FLT3 wild-type was calculated via GeneScan analysis [13] and the expression of NUP98-NSD1 was analyzed in 246 out of 353 patients with available material by real-time quantitative PCR using previously described primers [19]. Initial analysis demonstrated that the selected cohort was representative for all patients treated between 2004 and 2017 on the AML-BFM protocols for features such as gender, age, AML subtype, initial cytogenetics, and preliminary, early response to treatment (data not shown).

Clinical end-points were defined as previously described [28, 29] and survival rates were calculated via Kaplan-Meier analysis and compared by log-rank test. Multivariate analysis was performed using Cox regression model evaluating the hazard ratio (HR) of each covariate with 95% confidence interval (CI). Stem cell transplantation was included in the Cox regression model as a time-dependent variable. Differences with a p value less than 0.05 were considered as significant. Data were analyzed using the Statistical Analysis System software version 9.4 (SAS Institute, Cary, NC). Data acquisition was stopped at June 30, 2018, with a median follow-up of 3.6 years.

3. Results

3.1. Study Cohort and Patient Characteristics. In this study, we included 353 patients treated on either the AML-BFM 2004 or AML-BFM 2012 protocol for whom sufficient material and information were available (Figure I(a)). As shown in Table I, 48 (14%) patients had WT1 and 52 (15%) FLT3-ITD mutations in their leukemic blasts at diagnosis. Mutations in NPM1, NRAS, and c-KIT were present in the blasts of 9%, 17%, and 12% of patients, respectively. Most patients with mutated WT1 (n=35, 73%) harbored at least one co-occurring mutation in the AML blasts, with the most common being FLT3-ITD (n=19, 40%) followed by NRAS mutations (n=11,
AML-BFM trials 2004 - 2017
0 - 18 years old
de novo AML (n = 1034)

FAB M3 AML
Yes
excluded n = 193

Down Syndrome
No
included n = 841

available material
available data
No
excluded n = 488

included n = 353

Known status of NUP98-NSD1
No
excluded n = 107

included n = 246

known status of FLT3-ITD AR
No
excluded n = 9

included n = 237

(a) Study flowchart

(Features)

Figure 1: Study flowchart and patient characteristics. (a) Study flowchart outlining the process of patient recruitment in the data analysis. (b) WT1 mutations often co-occurred with FLT3-ITD and other genetic aberrations. AML-BFM, acute myeloid leukemia-Berlin-Frankfurt-Muenster; n, number; WT1, Wilms Tumor 1; FLT3-ITD, fms-related tyrosine kinase 3-internal tandem duplication; NPM1, nucleophosmin 1; NRAS, neuroblastoma RAS viral oncogene homolog; c-KIT, KIT proto-oncogene; CBF, core binding factor; MLL, rearrangements of MLL gene; NUP98-NSD1, Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1 fusion gene; CN, cytogenetic-normal AML; AR, allelic ratio; CCR, continued complete remission; LFU, lost to followup; NR, non-response; PR, partial remission. Other cytogenetic aberrations include translocation of chromosomes 8 and 21 and inversion or translocation of chromosome 16. Other cytogenetic aberrations include translocation of chromosomes 8 and 21 and inversion or translocation of chromosome 16.
| FEATURE | WT1 | FLT3-ITD | NUP98-NSD1 | AUL / other | FLT3-ITD status | NUP98-NSD1 status | AUL status |
|---------|-----|---------|------------|-------------|----------------|-----------------|------------|
| Number of patients | 100 | 100 | 100 | 100 | WT1 | FLT3-ITD | NUP98-NSD1 |
| WBC, x10^9/L | 30 | 58% | 153 | 52% | 133 | 48% | 133 |
| FLT3-ITD | 48% | 46% | 24% | 22% | 22% | 22% | 22% |
| WT1 | 153 | 52% | 133 | 48% | 133 | 48% | 133 |
| FLT3-ITD | 48% | 46% | 24% | 22% | 22% | 22% | 22% |
| NUP98-NSD1 | 50% | 50% | 24% | 22% | 22% | 22% | 22% |
| AUL | 22 | 6% | 16 | 5% | 13 | 5% | 13 |
| WT1 | 153 | 52% | 133 | 48% | 133 | 48% | 133 |
| FLT3-ITD | 48% | 46% | 24% | 22% | 22% | 22% | 22% |
| NUP98-NSD1 | 50% | 50% | 24% | 22% | 22% | 22% | 22% |
| AUL | 22 | 6% | 16 | 5% | 13 | 5% | 13 |
| WT1 | 153 | 52% | 133 | 48% | 133 | 48% | 133 |
| FLT3-ITD | 48% | 46% | 24% | 22% | 22% | 22% | 22% |
| NUP98-NSD1 | 50% | 50% | 24% | 22% | 22% | 22% | 22% |
| AUL | 22 | 6% | 16 | 5% | 13 | 5% | 13 |
23%, Table 1 and Figure 1(b)). Comparably, the majority of patients with FLT3-ITD had additional mutations in other genes (n=32, 62%), most commonly in WT1 (n=19, 37%) and NPM1 (n=11, 21%). Patients with mutated WT1 or FLT3-ITD were older compared to the rest of the study cohort, and AML FAB M1/M2 was the most common morphologic subtype in both groups (Table 1). In addition, the AML blasts of more than half of patients with WT1 (n=25/48, 52%) and FLT3-ITD (n=28/52, 54%) had a normal karyotype at diagnosis; these percentages were significantly higher than those in patients without mutations in each of the two genes (p<0.0001, Table 1).

3.2. Characteristics of WT1 Mutations. We identified 64 different WT1 sequence alterations in 48 patients (Table 2). These alterations were frequently located in exon 7 (n=55, 86%) and predominantly resulted in frameshifts producing premature termination codons (PTC’s). In total, nine single nucleotide variants (SNVs) were found, mostly in exon 9 (n=7, 78%). Only three of the nine SNVs were not previously reported as pathogenic (Table 2). Using NGS, we characterized multiple distinct WT1 mutations with highly diverse variant allele frequencies in 13 patients (11 patients had two and 2 patients, three distinct mutations). We then analyzed the heterozygosity of these mutations via the integrative genomic viewer (Broad Institute, MA, USA) and determined that they were all located on individual/different alleles/reads (Table 2).

3.3. Survival Significance of the Genomic Aberrations. Next, we analyzed the impact of each mutation on the clinical outcomes. Our analysis identified WT1 and FLT3-ITD, but not NRAS, NPM1, or c-KIT mutations as single factors that significantly increased the chance of relapse or treatment failure and reduced the probability of 3-year overall survival (OS) in our patient cohort (Figures 2(a), 2(b), and 3). In addition, FLT3-ITD but not WT1 mutations significantly decreased the 3-year probability of event-free survival (EFS, Figure 2(b)). When we grouped the two mutations together, the survival analysis revealed a 3-year EFS of 29±11% for patients with both WT1 and FLT3-ITD mutations compared to 63±3% for patients with none of these mutations (p=0.0004) and 61±11% or 45±9% for patients with only WT1 mutation (p=0.016) or FLT3-ITD (p=0.16), respectively (Figure 2(c)). Corresponding to this low EFS, co-occurrence of these two mutations was associated with an increased cumulative incidence of relapse (CIR) of 65±12% compared to 32±12% for patients with none of these mutations (p=0.002) and 39±11% or 46±9% for patients with only WT1 mutation (p=0.05) or FLT3-ITD (p=0.08), respectively (Figure 2(c)). Furthermore, we identified a low 3-year OS probability of 33±12% in patients with co-occurrence of WT1 and FLT3-ITD, which was significantly lower than those of patients without these mutations (81±3%, p<0.0001), patients with only mutated WT1 (87±7%, p=0.0007), and patients with only FLT3-ITD (67±9%, p=0.017, Figure 2(c)). Comparing the curves for EFS and OS clearly demonstrated that our second line treatment was not able to rescue any patient with co-occurrence of WT1 and FLT3-ITD mutations, while the OS rates increased by more than 20% for the other three subgroups (Figure 2(c)).

3.4. Impact of NUP98-NSD1 Fusion. To further characterize the prognostic significance of WT1 and FLT3-ITD mutations, we analyzed the expression of NUP98-NSD1 fusion in our patient cohort (Figure 1(a)). From 246 patients with available material for this retrospective real-time quantitative PCR analysis, 15 (6%) of them were identified to have the NUP98-NSD1 translocation. Most of these patients (12/15, 80%) harbored additional WT1 or FLT3-ITD mutations: 3 patients carried both WT1 and NUP98-NSD1, 4 had a co-occurrence of FLT3-ITD and NUP98-NSD1, and 5 patients carried all three genetic alterations (Figure 1(b)). Only 1 of these 15 patients had a previous known status of NUP98-NSD1 by conventional karyotyping: 2 others were previously diagnosed with deletion of chromosome 5, 1 carried an inversion of chromosome 16 (no other mutations and still in continuous complete remission), 4 carried complex karyotypes or rare aberrations, and 7 had no other cytogenetic abnormalities (data not shown).

We then analyzed the prognostic significance of NUP98-NSD1 in the cohort of 246 patients with the known status of this fusion gene (Figure 1(a)). As a single factor, the presence of NUP98-NSD1 in AML blasts of patients at diagnosis was associated with a significant increase in CIR (81%) in addition to decreased probabilities of 3-year EFS and OS (Figure 4(a)). Combining NUP98-NSD1 with WT1 and FLT3-ITD mutations in our multivariable survival analysis revealed that patients with all three or either two of these mutations had worse survival outcomes. These patients had a higher CIR of 73±11% compared to the CIR of 30±4% for patients with none of these aberrations or NUP98-NSD1 alone (p<0.0001) and the CIR of 37±13% or 38±10% for patients with only mutated WT1 (p=0.0078) or FLT3-ITD (p=0.013), respectively (Figures 4(a) and 4(b)). The increased CIR translated into a lower 3-year EFS probability of 23±10% for patients with triple or double mutations compared to the EFS of 62±4% for patients with none of these mutations or only NUP98-NSD1 (p<0.0001) and the EFS of 63±13% or 54±10% for patients with only WT1 (p=0.003) or FLT3-ITD (p=0.036) mutations, respectively (Figure 4(b)). Moreover, co-occurrence of all three or any double mutations resulted in a significantly lower 3-year OS probability of 42±12% compared to 80±8% for patients with none of the mutations or only NUP98-NSD1 (p=0.0003) and 88±8% or 73±10% for patients with only WT1 (p=0.0007) or FLT3-ITD (p=0.049) mutations, respectively (Figure 4(b)).

3.5. Survival Significance of the FLT3-ITD Allelic Ratio. We have previously established the prognostic significance of an FLT3-ITD allelic ratio of ≥0.4 in pediatric AML [12]. Therefore, to determine the impact of the mutational burden of FLT3-ITD on treatment outcomes in the present cohort, we calculated the FLT3-ITD AR in patients with available data/material. As indicated in Figure 1(b), 27 patients had an AR ≥0.4 at diagnosis. Analyzing the survival impact of
Table 2: Characteristics of WT1 Variants.

| UPN | exon | seq. read | mutation sequence | amino acid alteration | VF (%) | dbSNP or COSMIC ID | published | previously reported sample | outcome |
|-----|------|-----------|------------------|----------------------|--------|-------------------|-----------|----------------------------|---------|
|     |      |           |                  |                      |        |                   |           |                            |         |
| **missense substitutions** |     |           |                  |                      |        |                   |           |                            |         |
| 8   | 9    |           | c.1333C>T        | p.Arg445Trp          | 19.1   | rs121907900, COSM21417 | Yes       | WT                         | CCR     |
| 15  | 9    |           | c.1345C>A        | p.Leu449Met          | 5.49   |                   | No        | CCR                        |         |
| 20  | 9    |           | c.1385G>A        | p.Arg462Gln          | 47.21  | rs121907903, COSM4191067 | Yes       | AML, colon cancer, adenocarcinoma | CCR     |
| 21  | 9    |           | c.1343A>G        | p.His448Arg          | 33.12  |                   | Yes       | AML, mesothelioma           | CCR     |
| 23  | 9    |           | c.1333C>T        | p.Arg445Trp          | 72.42  | rs121907900       | Yes       | WT, DDS                    | CCR     |
| 26  | 9    |           | c.1097C>G        | p.Ser366Cys          | 2.57   |                   | No        | CCR                        |         |
| 35  | 9 different |           | c.1334G>A       | p.Arg445Gln          | 3.12   | rs121907903, COSM4191067 | Yes       | AML, colon cancer, adenocarcinoma | Relapse |
|     | 9 different |           | c.1307G>A      | p.Cys436Tyr          | 44.21  |                   | Yes       | AML                        | Relapse |
| **nonsense substitutions/insertions, deletions or duplications** |     |           |                  |                      |        |                   |           |                            |         |
| 1   | 7    |           | c.1090_1093dupTC | p.Ala365Valfs*4      | 43     |                   | Yes       | AML                        | CCR     |
| 2   | 7    |           | c.1048-4_1056dupGCAGGAGATGGCGGA | p.Arg353Alafs*19 | 30.25  |                   | No        | LFU in CCR                 |         |
| 3   | 7    |           | c.1087_116dup74  | p.Lys387Asnfs*44     | 44     |                   | No        | Relapse                    |         |
| 4   | 7 different |           | c.1087_1091dupCGGTCA | p.Ala365Glyfs*69 | 5.08   |                   | Yes       | AML, T-ALL                 | Relapse |
| 4   | 7 different |           | c.1091C>A       | p.Lys387Asnfs*69     | 28.08  |                   | Yes       | AML, WT                    | Relapse |
| 5   | 7    |           | c.1083_1098delTGTACGGTGCGGCATCT | p.Val362Argfs*65 | 46.82  |                   | No        | NR/PR                      |         |
| 6   | 7    |           | c.1059dupT      | p.Val354Cysfs*14     | 35.9   |                   | Yes       | AML                        | Relapse |
| 7   | 7    |           | c.1179dupG      | p.His394Alafs*8      | 25     |                   | No        | CCR                        |         |
| 9   | 7 different |           | c.1078_1079insGCCGA | p.Thr360Serfs*74   | 38.7   |                   | No        | NR/PR                      |         |
|     | 7 different |           | c.1084_1085insGC | p.Val362Glyfs*71     | 52.9   |                   | No        | CCR                        |         |
| 10  | 7    |           | c.1074_1077delCCCG | p.Thr360Profs*9     | 9.9    |                   | No        | CCR                        |         |
| 11  | 7    |           | c.1079_1090delCTCTGTACGGTGTCATGTCA | p.Thr360Metfs*5 | 55.23  |                   | No        | CCR                        |         |
| 12  | 7    |           | c.1058_1059insGA | p.Val354Metfs*5      | 31.6   |                   | No        | CCR                        |         |
| 13  | 7 different |           | c.1058_1059insGGTGTTG | p.Pro355Cysfs*14  | 5.6    |                   | No        | AML                        | Relapse |
|     | 7 different |           | c.1078_1084dupACCTTGG | p.Val362Alafs*16 | 22.81  |                   | Yes       | AML                        | CCR     |
| 14  | 7    |           | c.1090_1093dupTGG | p.Ala365Valfs*4     | 22.81  |                   | Yes       | AML                        | CCR     |
| 16  | 7    |           | c.1054_1058dup   | p.Val362Alafs*16    | 73     |                   | No        | CCR                        |         |
| 17  | 7    |           | c.1087delCCGGG   | p.Arg363Glyfs*17    | 24.3   |                   | No        | CCR                        |         |
| 18  | 7    |           | c.1054_1055insF  | p.Arg363Leufs*16    | 67.2   |                   | Yes       | T-ALL                      | CCR     |
| 19  | 7    |           | c.1077_1081insTGTCCTTCCGCCAG | p.Thr360Cysfs*13 | 36.95  |                   | No        | Relapse                    |         |
| 22  | 7    |           | c.1087delCCGGG   | p.Arg363Glyfs*5     | 41.88  |                   | Yes       | AML                        | CCR     |
| 24  | 7    |           | c.1083_1090dupTGACGGT | p.Ser364Leufs*71 | 3.8    |                   | Yes       | AML                        | CCR     |
| UPN | exon | seq. read | mutation sequence<sup>a</sup> | amino acid alteration | VF (%) | dbSNP or COSMIC ID | published | previously reported sample | outcome |
|-----|------|----------|--------------------------------|----------------------|--------|-------------------|-----------|----------------------------|---------|
| 25  | 9    | different | c.1323,1338dupAAAGTTCTCCGGTCC  | p.Asp447Lysfs*18     | 40.1   | No                | No        | CCR                        |         |
|     | 9    | different | c.1322,1332dupGAAGTTCTCC       | p.Arg445Glufs*9      | 40.5   | No                | No        |                            |         |
| 27  | 7    | different | c.1077,1078insGGTGTG            | p.Thr360Valfs*9      | 43.71  | No                | No        |                            |         |
|     | 7    | different | c.1089dupG                     | p.Ser364Valfs*4      | 49.24  | No                | Yes       | AML                        | CCR     |
| 28  | 7    | different | c.1086delKinsCCA                | p.Arg353Profs*6      | 19.72  | No                | No        |                            |         |
|     | 7    | different | c.1054,1055insAAAAGAATT        | p.Arg352delins4      | 19.55  | No                | No        |                            |         |
| 29  | 7    |           | c.1079dupG                     | p.His394Alafs*8      | 2.5    | No                | No        |                            |         |
| 30  | 7    |           | c.1048,1057delGATGTG GCCGAGinsAAGG | p.Asp350, Arg353    | 46.34  | No                | No        |                            |         |
| 31  | 7    |           | c.1093dupG                     | p.Ala365Glyfs*3      | 44.49  | Yes               | AML       |                            |         |
| 32  | 7    |           | c.1048-8,1055dupGCCTGAGGGATGTCG | p.Arg353Profs*20    | 2.5    | No                | No        |                            |         |
| 33  | 7    |           | c.1090,1091dupTC               | p.Ala365Argfs*68     | 44.25  | COSM28955         | Yes       | AML                        | Relapse |
| 34  | 7    | different | c.1087delGinsGA                | p.Arg363Glufs*5      | 4.6    | No                | No        |                            |         |
|     | 7    | different | c.1086dupA                     | p.Arg363Thrfs*5      | 5.33   | Yes               | AML       |                            |         |
|     | 7    | different | c.1090,1093dupTC              | p.Ala365Valfs*4      | 36.29  | Yes               | AML       |                            |         |
| 36  | 7    | different | c.1090,1093dupTCGG            | p.Ala365Valfs*4      | 6.94   | Yes               | AML       |                            |         |
|     | 7    | different | c.1091dupC                    | p.Ala365Glyfs*3      | 39.42  | Yes               | AML       |                            |         |
| 37  | 7    | different | c.1057delGinsGG                | p.Arg353Glyfs*15     | 42.78  | Yes               | AML       |                            |         |
|     | 7    | different | c.1087delGinsGG                | p.Ala365Glyfs*70     | 52.11  | No                | No        |                            |         |
| 38  | 7    |           | c.1068,1076delAGTAGCCCGGinsGACGGTGCGTATTATA | p.Val357Thrfs*77    | 42.34  | No                | No        |                            |         |
| 39  | 7    |           | c.1087delGinsGG                | p.Arg363Glyfs*5      | 47.54  | Yes               | AML       |                            |         |
| 40  | 7    | different | c.1058,1059insGGTCGCGTCGGCG    | p.Gly356Leufs*6      | 48.49  | No                | No        |                            | Early Death |
|     | 7    | different | c.1082,1091dupITGTACGGTC      | p.Ala365Cysfs*6      | 41.83  | Yes               | AML       |                            |         |
| 41  | 7    | different | c.1123dupA                    | p.Met357Asns*9       | 44.5   | Yes               | AML       |                            |         |
|     | 7    | different | c.1057,1058insTA              | p.Arg353Leufs*6      | 45.8   | No                | No        |                            |         |
| 42  | 7    |           | c.105L,1055dupTGTCG            | p.Arg353Cysfs*7      | 34.73  | No                | No        |                            | Relapse |
| 43  | 7    |           | c.1058delGinsCC                | p.Arg353Profs*15     | 44.78  | Yes               | COSM28946 | AML                        | Relapse |
| 44  | 7    | different | c.1079,1080delinsGA            | p.Thr360Argfs*4      | 20.37  | No                | No        |                            | Relapse |
|     | 7    | different | c.1088,1089insCCTCGG         | p.Ala365Glyfs*6      | 10.69  | No                | No        |                            | Relapse |
| 45  | 7    |           | c.1090,1091insAGGT            | p.Ser364fs*1         | 42.97  | No                | No        |                            | Relapse |
| 46  | 7    |           | c.1058delGinsCC                | p.Arg353Profs*15     | 51.08  | Yes               | COSM28946 | AML, T-ALL                  | NR/PR   |
| 47  | 7    | different | c.1048-3,1055dupCAGAGTGTCG    | p.Val354Metfs*8      | 2.49   | No                | No        |                            |         |
|     | 7    | different | c.1053dupG                    | p.Arg352Alafs*16     | 3.83   | Yes               | COSM28980 | AML                        |         |
|     | 7    | different | c.1054delGinsGG               | p.Arg352Glyfs*16     | 35.86  | Yes               | COSM28970 | AML, T-ALL                  |         |
| 48  | 7    |           | c.1089,1090insGGGCCTCTTGATGCG  | p.Ser364Glyfs*73     | 40.49  | No                | No        | Relapse                    |         |

<sup>a</sup>Transcript ID: NM_000378 was used to describe all alterations.

UPN, unique patient number; Seq. read, sequence read; VF, variant allele frequency; dup, duplication; ins, insertion; indel, insertion-deletion; fs, frame-shift; *termination codon; WT, Wilm's tumor; DDS, Denis-Drash syndrome; T-ALL, T-cell acute lymphoblastic leukemia; CCR, continued complete remission, NR, non-response; PR, partial response; LFU, lost to follow-up.
the FLT3-ITD AR ≥0.4 revealed that as a single factor, it was associated with an EFS of only 25±8% and an OS of only 47±10%, respectively (Figure 5(a)). Remarkably, the co-occurrence of FLT3-ITD AR ≥0.4, WT1, and NUP98-NSDI as triple or double mutations significantly increased the CIR to 93±15% compared to the CIR of 31±4% for patients with no mutations or only NUP98-NSDI or FLT3-ITD AR <0.4 (p<0.0001) and to the CIR of 31±11% or 36±15% in patients with only WT1 (p<0.0001) or FLT3-ITD AR ≥0.4 (p=0.001) mutations, respectively (Figure 5(b)). The probability of 3-year EFS was zero in patients with double or triple WT1, FLT3-ITD AR ≥0.4, and NUP98-NSDI mutations as opposed to 61±4% in patients with no mutations or only NUP98-NSDI or FLT3-ITD AR <0.4 (p<0.0001) and 69±11% or 45±15% for patients with only mutated WT1 (p<0.0001) or FLT3-ITD AR ≥0.4 (p=0.019), respectively (Figure 5(b)). Finally, the co-occurrence of double or triple mutations resulted in a 3-year OS probability of 27±13%, which was significantly lower than the 3-year OS of 79±3% in patients with no mutations or only NUP98-NSDI or FLT3-ITD AR <0.4 (p<0.0001) and 90±7% or 73±13% in patients with only WT1 (p=0.0003) or FLT3-ITD AR ≥0.4 (p=0.06) mutations, respectively (Figure 5(b)). By multivariate analysis including WT1 mutation, FLT3-ITD AR ≥0.4, core-binding factor aberrations, early bone marrow response to treatment, and stem cell transplantation as covariables, we confirmed that the interaction of these three factors, and not each of the aberrations individually, was a significant predictor of poor prognosis for EFS (p=0.008, HR: 3.88, 95% CI: 1.42 – 10.6) and OS (p=0.042, HR: 3.42, 95% CI: 1.04 – 11.21, Table 3). Importantly, none of the patients with triple mutations survived and the only patients who could be rescued harbored

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**Figure 2:** Co-occurrence of WT1 and FLT3-ITD mutations at initial diagnosis of pediatric AML predicts poor survival outcomes. (a) WT1 mutation as single factor increased the incidence of relapse, reducing the probability of survival. (b) The presence of FLT3-ITD, individually, leads to an increased chance of relapse and decreased patient survival. (c) Clinical consequences of WT1 and FLT3-ITD were dependent on each other. WT1, Wilms Tumor 1; FLT3-ITD, fms related tyrosine kinase 3-internal tandem duplication; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; n, number. *No response to treatment was considered as the occurrence of an event at time zero.
double NUP98-NSD1 and WTI or NUP98-NSD1 and FLT3-ITD mutations (Figure 1(b)), thus resulting in an OS of 27±13% (Figure 5(b)).

4. Discussion

Treatment of pediatric AML has significantly improved over the past three decades due to the development of intensified first-line treatments, efficient second-line therapies, and optimized supportive care [2, 30]. The success is, at least partly, achieved by more efficient risk group stratification using factors such as somatic mutations and cytogenetic aberrations of AML blasts at diagnosis as well as considering the primary response to treatment to optimize the allocation of patients to standard or enhanced treatment options [1]. In the present study, we analyzed the influence of three parameters, mutations in WTI and FLT3 and the translocation of NUP98-NSD1, on the outcome of pediatric patients in the German AML-BFM 2004 and 2012 protocols. Although all three parameters have been established by us and others as important prognostic factors in both pediatric and adult patients [8–14, 20–22], their combined utility to identify high-risk patients likely to experience dismal treatment results has not yet been reported in a contemporary pediatric AML trial.

In a cohort of 237 patients treated within the AML-BFM 2004 and 2012 protocols and with sufficient material for re-analysis, we observed favorable outcomes for 3-year EFS of 61% and 69% and OS of 79% and 90% in patients

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**Figure 3: Mutations in NPM1, NRAS, and c-KIT had no impact on survival.**

(a) Prognostic impact of mutated NPM1 on EFS, OS, and CIR. (b) Prognostic impact of mutated NRAS on EFS, OS, and CIR. (c) Prognostic impact of c-KIT mutation on EFS, OS, and CIR. NPM1, nucleophosmin 1; NRAS, neuroblastoma RAS viral oncogene homolog; c-KIT, KIT protooncogene; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; n, number. *No response to treatment was considered as the occurrence of an event at time zero.*
Figure 4: Prognostic significance of NUP98-NSD1 fusion. (a) NUP98-NSD1 as single factor predicted poor outcomes. (b) Inclusion of NUP98-NSD1 as poor prognostic factor with WT1 mutation and FLT3-ITD, predicted poor outcomes for patients harboring all three factors in addition to patients with NUP98-NSD1 and WT1 mutation or FLT3-ITD. Patients with unknown status of NUP98-NSD1 fusion were excluded from this analysis. WT1, Wilms Tumor 1; FLT3-ITD, fms related tyrosine kinase 3-internal tandem duplication; NUP98-NSD1, Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1 fusion gene; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; mut, mutated; pos, positive; neg, negative. *No response to treatment was considered as the occurrence of an event at time zero. **Three patients with NUP98-NSD1 are included in this group.
Figure 5: Prognostic significance of mutational burden of FLT3-ITD. (a) FLT3-ITD with an allelic ratio ≥0.4 as a single factor predicted poor outcomes. (b) High mutational burden of FLT3-ITD was another predictor of poor prognosis when it occurred with WTI and/or NUP98-NSD1. Patients with an unknown FLT3-ITD AR were excluded from this analysis. NUP98-NSD1, Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1 fusion gene; FLT3-ITD, fms related tyrosine kinase 3-internal tandem duplication; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; AR, allelic ratio; SE, standard error; n, number. *No response to treatment was considered as the occurrence of an event at time zero. **Three patients with NUP98-NSD1 are included in this group.
without WT1 mutations or NUP98-NSD1 fusion or with only one of these factors. Patients with leukemic blasts that were FLT3-ITD positive but negative for WT1 and NUP98-NSD1 mutations and that had an FLT3-ITD AR ≥ 0.4 still achieved an EFS of 45% and an OS of 73%. Surprisingly, our data therefore suggests that without WT1 and NUP98-NSD1 mutations, the negative impact of FLT3-ITD even with an AR ≥ 0.4 might not be as severe as previously published [12, 17]. However, all patients positive for at least two of the three risk factors and with an FLT3-ITD AR ≥ 0.4 had events within the first three years and only 27% could be rescued by our salvage therapies. These unfavorable results in our double or triple mutated group unequivocally demonstrate that our current first-line treatment strategies for these patients are still insufficient/inadequate and urgently need improvement.

Of the three risk factors, currently only the FLT3-ITD mutation can be specifically targeted with inhibitors [31]. Although the first generations of these drugs only achieved limited and often transient efficacy due to intrinsic and extrinsic adaptations in the AML blasts and/or the environment [31], combination therapies of newer tyrosine kinase inhibitors such as Quizartinib with standard chemotherapy seem to be relatively well tolerated and in initial studies have demonstrated survival improvement in relapsed or refractory AML patients [32–34]. Due to the important role of FLT3 pathway activation in AML, numerous combinations of FLT3 inhibitors with other drugs are currently being tested. Whether these results will also be helpful for the treatment of pediatric AML will need to be carefully determined in future studies, especially considering the clonal heterogeneity of FLT3-ITD and the additional survival burden that it causes by increasing drug resistance through clonal evolution or selection and further expansion of resistant AML clones [35, 36]. Nevertheless, it is tempting to speculate that the simple addition of a newer FLT3 inhibitor to our standard therapy might be a feasible, well-tolerated, and effective approach for all patients with blasts that are positive for the FLT3-ITD mutation, regardless of the status of alterations in WT1 or NUP98.

The role of WT1 in patients with AML is still controversial [4]. Although WT1 is overexpressed in the majority of leukemias and can be used as a marker for minimal residual disease and maybe even vaccination attempts, the prognostic and therapeutic relevance of high or absent WT1 expression levels is not unequivocally accepted [37–39]. In contrast, mutations in WT1 are clearly identified as determinants of poor prognosis and, as we showed here, confer a dismal prognosis especially in combination with FLT3-ITD or NUP98-NSD1 fusion. In the present study, we identified 64 monoallelic WT1 sequence alterations in exon 7 or exon 9 in the leukemic blasts of 48 patients. The majority of these alterations leads to frameshifts and/or premature terminations codons and thus shortened proteins. These mutant proteins can act in a dominant negative manner [40], which may contribute to a myeloid differentiation block present in AML blasts [41]. However, similar mutations have also been described in the context of Wilms tumors as gain-of-function mutations promoting proliferation [42]. Here, we show a favorable prognosis for patients with single WT1 mutations, with 26 out of 29 cases reaching continued complete remission (CCR) (Figure 1(b)). Therefore, based on a 3-year EFS of 69% and an OS of 90%, the development of

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**Table 3: Multivariate analysis.**

| Parameters                        | Hazard ratio | 95% confidence interval |
|-----------------------------------|--------------|-------------------------|
| WT1 mutation                      | 0.79         | 0.41 – 1.53             | 0.479 |
| FLT3-ITD AR ≥ 0.4                 | 1.55         | 0.69 – 3.51             | 0.288 |
| WT1 mutation, FLT3-ITD ≥ 0.4 and NUP98-NSD1 interaction | 3.88 | 1.42 – 10.66 | 0.008 |
| (t(8;21) and/or inv(16)           | 0.51         | 0.27 – 0.96             | 0.037 |
| Unsatisfactory early response to treatmenta | 1.31 | 0.79 – 2.18 | 0.294 |
| HSCTb                           | 0.25         | 0.1 – 0.64              | 0.004 |

WT1, Wilms tumor 1; FLT3-ITD, fms related tyrosine kinase 3-internal tandem duplication; NUP98-NSD1, Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1 fusion gene; t, translocation; inv, inversion; HSCT, hematopoietic stem cell transplantation.

a Un satisfactory early response to treatment was defined as persistence of >5% blasts in bone marrow at day 15 and/or 28 after treatment. b Hematopoietic stem cell transplantation events at first complete remission or after no response to other treatments were included in the multivariate analysis as a time-dependent variable.
new treatment approaches is not as urgently needed for these patients with WT1 mutated blasts that do not harbor FLT3-ITD or NUP98-NSD1 mutations.

Among the 31 different fusion gene partners of NUP98 identified so far, the NUP98-NSD1 (t(5:11) translocation is the most frequent and present in 4-7% of patients in pediatric AML patients [20–22]. Importantly, the NUP98 translocations that occur in AML all share the N-terminus of the protein and are thought to initially lead to epigenetic dys-regulation of different leukemia-associated genes including HOXA7, HOXA9, and HOXA10 in myeloid precursor cells [20]. Additional somatic mutations in other genes occur as secondary events and promote malignant transformation and uncontrolled cell growth [20]. As also shown in our patient data set, these secondary alterations often include activating mutations in FLT3 (FLT3-ITD) or truncating mutations in WT1 [21]. Strikingly, only three patients in our study had a NUP98-NSD1 translocation without mutations in FLT3 or WT1; two of these patients achieved and remained in first CCR at the end of data acquisition. The third patient had no other genetic risk factors but a very high initial white blood cell count of almost 400,000 cells/µl. Complete remission induction was delayed, and the patient relapsed a year later but was successfully treated by allogeneic stem cell transplantation with a follow-up of 10 years. Therefore, as also described previously [21], our patients with NUP98-rearranged blasts with WT1 and/or FLT3-ITD mutations had a poor prognosis, especially in contrast to patients with only WT1 and FLT3-ITD mutations, who could at least partially be rescued by allogeneic transplantation. However, due to the high risk of failure of the first-line treatment, stem cell transplantation already in first CCR seems to be an attractive option for cases of NUP98-rearranged AML [21, 22]. Nevertheless, it should be noted that even allogeneic stem cell transplantation is not always effective in improving the treatment outcome in patients with a high probability of treatment failure based on risk stratification. Thus, introducing novel treatment approaches such as the use of small inhibitors, e.g., venetoclax and isadananulin [43] or cellular therapies with allogeneic NK-cells or engineered T-cells with chimeric antigen receptors (CARs) [44] targeting leukemic blasts harboring NUP98 rearrangement or WT1 mutations should be taken into consideration in future clinical studies.

Recent analysis from a collaborative study between the American and Dutch children oncology groups (COG and DCOG) included patients from three clinical COG/DCOG trials and also young adults less than 39 years of age in the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) AML initiative [45]. Analysis of the different cohorts revealed similarly unfavorable outcomes with an EFS of 14-25% and an OS of 15-40% for patients with FLT3-ITD and WT1 mutations and/or the NUP98-NSD1 translocation [45]. In contrast to our findings however, the authors reported an EFS range of 15-35% in patients with FLT3-ITD only, which is lower than that achieved with current protocols, for which an EFS of 45% and an OS of 73% were found for patients with FLT3-ITD only. Notably, in the American-Dutch study, patients with co-occurrence of NPM1 mutations and FLT3-ITD (and without WT1 and NUP98-NSD1) were separated from patients with FLT3-ITD only and had a slightly increased, albeit probably not statistically significant, survival. Similarly, we have previously observed favorable outcomes for patients with NPM1 mutations in their AML blasts with normal karyotype and proved this impact was not affected by the presence of FLT3-ITD [46]. In the current cohort, five patients were positive for mutations in FLT3-ITD and NPM1 and negative for WT1 and NUP98 alterations. At present, four patients with a normal karyotype are still in first CCR, and the fifth patient with a complex karyotype and an FLT3-ITD AR >11 experienced early death. In summary, the principle findings of this American-Dutch study and the present study are very similar. However, the treatment outcomes for our patient groups are superior, most likely due to the fact that we included only patients between 0 and 18 years of age treated in Germany according to two contemporary protocols from the AML BFM study group.

5. Conclusion

Despite the fact that our study was partly based on data collected prospectively since 2004 and partly on data assessed de novo on stored material by either NGS or PCR, we can safely conclude that co-occurrence of the three factors, mutated WT1 and FLT3-ITD and/or NUP98-NSD1 translocation, still defines a subgroup of AML patients with devastating EFS and OS outcome, even with our current treatment protocols. Although the number of pediatric AML patients available for analysis of these three risk factors was limited and therefore not all interesting factors could be assessed in multivariate analysis, it is obvious that patients with double or triple mutations benefitted very little from the improved EFS and OS in our AML-BFM studies in recent years. Thus, for these pediatric patients, new and more targeted approaches are urgently needed for both first- and second-line treatments.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the current work. Dirk Reinhardt has consulting or advisory roles for Roche, Celgene, Hexal, Pfizer, Novartis, Boehringer and receives research funding from Celgene. Dirk Reinhardt received travel grants from Jazz Pharmaceuticals and Griffols. Naghmeh Niktoreh and Christine von Neuhoff received travel grants from Jazz Pharmaceuticals. The other authors have nothing to declare.

Authors’ Contributions

Helmut Hanenberg and Dirk Reinhardt contributed equally. Naghmeh Niktoreh and Christiane Walter collected and assembled data; Martin Zimmermann, Naghmeh Niktoreh,
Christiane Walter, Helmut Hanenberg, and Dirk Reinhardt analyzed and interpreted data; Naghmeh Niktoreh and Helmut Hanenberg wrote the manuscript; and all authors gave final approval of manuscript.

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